Vol. 25, No. 1, August-September, 1960

Russian Original Dated January-February, 1960

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BIOCHEMISTRY

A translation of BIOKHIMIYA, a publication of the Academy of Sciences of the USSR

Russian Original Dated January-February, 1960

Vol. 25, No. 1, pp. 1-140 August-September, 1960

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The National Institutes of Health of the Public Health Service has supported in part the cost of publishing BIOCHEMISTRY through the purchase of copies for distribution to American medical libraries.

BIOCHEMISTRY AND DARWINISM

Biochemistry and the Solution of the Problems of Evolution

Translated from Biokhimiya Vol. 25, No. 1, pp. 3-11, January-February, 1960

1. The Place of Biochemistry in Solution of Evolutionary Problems

An exhaustive solution of the problems of evolution can be reached only on the basis of a synthesis of the data from those branches of biology that study the organism from various viewpoints. Within the limits of the field of biochemistry we find the study of important changes in molecular structure of the chief forms of life and of biochemical processes during their evolution. Therefore biochemistry should be given a major place among the disciplines which study the evolutionary process from a different angle. However, it is not permissible to consider the possibilities from biochemical investigations alone (see the critical survey of attempts to solve the problem of evolution on the basis of biochemical materials alone [1]).

As objects of comparative study for biochemical changes in organisms during evolution we usually use contemporary organisms (and recently also, viruses) which are in various stages of evolutionary development. However, though biochemistry has had to overcome great difficulties in accomplishing it, it has not given up the study of organisms which lived earlier, and paleobiochemistry exists [2, 3]. The field of paleobiochemistry is the study of preserved remains of organisms (which can be studied morphologically) with corrections for possible chemical changes since the time of death.

Obviously, the first work in this field will be chemical investigation of skeletons [2]. We also note the serological study of the blood of the Siberian mammoth preserved in ice for about 25,000 years [4], mummies aged about 5000 years [5], and parts of human skulls from the Pleistocene which have lasted 10,000 years or more [6]. An important place is occupied by recent chemical studies of the amino acid composition of the shells of fossil mollusks from the Miocene (age of the objects about 25,000,000 years [7]). Related to this is the study of remains which have not kept their original form but have left breakdown products of the organisms.

It is also apparent that the developing science of the emergence of life on earth [8] belongs to paleobiochemistry, since it is concerned with the origin and development of the organisms which existed earlier. We also note the new approach [9] which uses the study of the nature of breakdown processes in organisms in different geological epochs insofar as these processes are related to the activities of other living organisms to trace the changes in biochemical activities of these other organisms in the corresponding epoch. This method has been used to study changes in the biochemical activities of microorganisms and lower plants during evolution on the basis of analysis of conditions of formation and chemical composition of caustobiolities. Thus, paleobiochemistry is linked with biogeochemistry and geochemistry as a whole.

It is true that the possibilities of paleobiochemistry are much more limited than are those of the biochemistry of living organisms, but the facts established by paleobiochemistry are of value and can help to reconstruct the picture of biochemical changes of organisms in the course of evolution. However, the main role in the solution of these problems is played in any case by the comparative biochemistry of living organisms with different degrees of evolutionary development, though we must consider the possible changes of these organisms from the time of their origin. As yet this field of biochemistry which is directly related to evolutionary problems has not been fully formulated, and comparative biochemical data are obtained not as a result of special investigations only, but more often as side results of some other biochemical study.

Comparative biochemical study of organisms has been carried out at various levels and has explained the following in their comparative aspects: 1) Total chemical composition of organisms – the origin of one or another group of substances and their quantitative ratios (see, for example, comparisons for animal organisms [10]); 2) the chemical structure of substances of different types in the organism – general characteristics and varying structures (see comparisons for proteins [11-19], nucleic acids [20, 21], fats [22-24], carotenoids [25]); 3) distinct biochemical processes which lie at the basis of physiological function – nervous activity [26-28], movement [28, 29], vision [30], bioluminescence [31], thyroid hormone function [32] – these investigations merge with the field of comparative physiology; 4) enzymes and enzyme systems which determine the course of various biochemical processes in the organisms being compared (we mention the work on comparison of the "quality" of the enzymes [33] and the chemical structure of enzymes, especially phosphoglyceraldehyde dehydrogenase, α-amylase, and lysozyme, isolated in pure form from various organisms [34]).

Where morphology fails because of the absence of homologous (or analogous) organs in the organisms being compared, the biochemist continues his investigations by comparing substances, biochemical processes, enzymes, and enzyme systems which lie at the basis of

physiological functions. As a first example [31, 30]: Biochemical investigation has shown that contractile proteins isolated not only from the muscles of various animals, but also from the contractile structures of other organisms have a single type of enzyme action (adenosine triphosphatase activity) and structural changes produced by the action of ATP are one more example of the unity of evolutionary origin of organisms. As a result of these studies ideas of change in contractile function during evolution have been greatly extended, including processes which are involved in such different motions as the movement of mimpsa leaves, ameboid movement, motion of cilia, of flagellates, and also the motion of the tails of spermatozoa and the flow of protoplasm in myxomycetes. All the problem of the change of this function during evolution are presented in a wide, truly Darwinian light, for which morphology alone is insufficient. As a second example [31]: Biochemical study has shown that the same system, acetylcholine-choline esterase plays an important part in the electrical activity and stimulation not only of the nervous system in animals, but also in those organisms in which differentiation of the nervous system has not yet occurred (in the "prenerve" stage of evolution); it is on this system that the characteristic effect of electrical stimulation for paramecium depends. Here also ideas on the developing function of the nervous system and the changes during evolution extend beyond those which can be given by morphology.

Thus the possibilities of biochemical demonstrations are greater than are those of morphology. However, when biochemistry does not cast new light on some evolutionary problem and can only confirm its solution which has already been reached from other results, the use of biochemistry can often be of value. Thus, for example, for the formulation of evolutionary ideas, especially among biochemists themselves, it would seem there is still need for additional material to confirm from a different viewpoint the great arsenal of morphological, embryological, paleontological and other evidence for evolution. Another example: Some authors have presented a series of biochemical facts which confirm biogenetic laws [35, 36] and also the law of irreversibility of evolution [37, 36]. These laws in their day were clear enough from morphological data, yet the presentation of biochemical facts provided a new viewpoint and gave a new illustration of these laws which deepened our understanding of them.

2. Biochemistry and the Problem of Phylogeny

The Common Evolutionary Origin of Organisms. For a long time we have known the presence of substances of the same groups in organisms, independent of their stage of evolutionary development, including chiefly proteins, carbohydrates, and fats. This was certainly considered by Darwin himself. Thus, in developing the idea of the origin of organisms "from one original form," "from one prototype," Darwin noted among the number of other arguments that "all living beings have much in common among themselves in chemical composition" [38]. It has also been noted that, on the basis of function of organisms in various stages of evolution, there are common biochemical processes, such as respiration and fermentation [39-41].

The fact of common chemical composition and biochemical processes in organisms continues to remain important for an understanding of the unity of their evolutionary origin, and should lead to a number of important pieces of evidence for evolution and its monophyletic explanation. Actually, these facts can be explained only in the light of evolutionary doctrine [1, 42]; evidently the aggregation of substances which compose the material basis of organisms, and the chief biochemical mechanisms were produced during the formation of the first primitive organisms and their transformations with the development of simple, one-celled organisms (actually this stage of evolution is characterized by the greatest biochemical gains). The completeness of the vital functions of the organism depends on their functioning within the limits of its simple reactions with the surrounding medium. Further, internal medium is formed which guarantees the establishment for the cells of conditions to which they were never adapted in an external environment. This does not require a fundamental change in the biochemical mechanisms of the cells; these mechanisms lie at the basis of the biochemical activities of organisms with many cells and changes in the biochemical mechanisms of the cells during evolution occur without disrupting the line of reciprocal adaptation of the cells and their specialization as they differentiate depending on the function which they perform. The fact that the biochemical activity of many one-celled organisms is complicated, so that in addition to the ordinary functions they produce substances with structures unusual for organisms with many cells (such as D-amino acids, fatty acids with branched chains, specific antibiotics, etc.) must be explained by the fact that these one-celled organisms, which have not proceeded along the line of uniting cells, have followed the path of direct adaptation to the surrounding medium by deeper changes in biochemical mechanisms.

Divergent Character of Evolution. Phylogenetic Relations between Organisms. The accumulation of biochemical knowledge has shown, along with similarities in chemical composition and biochemical activities, also differences in organisms of different systematic subdivisions with different morphological structures. These differences are the result of divergencies in the course of evolution which are illustrated by the character of the different biochemical signs (the same as for the morphological ones) in the "hierarchical" order of the systematic subdivisions.

In a number of cases data on the chemistry of organisms make the systematic position of organisms of various subdivisions more precise and correct conclusions made on the basis of morphological data only; we have a

"chemical taxonomy," a "chemical system" (see the history of the development and principles applied to plants [43-45]) which must be considered as part of the systematic treatment of organisms.

The help of biochemistry in explaining the phylogenetic relations will become more real when we will be able to establish more clearly the paths of change in the chemical structure of substances and in biochemical activity during the course of evolution. General considerations still predominate in this field. It is assumed [46] that changes in the "physiological-chemical signs," during the evolution of plants are characterized by simple chemical relations (oxidation, methylation, acetylation, benzoylation, etc.).

The idea [47] has been expressed that the organism as a result of successive mutations can acquire the enzymes needed for the synthesis of complex substances from simple substances as precursors for the synthesis of complex substances. This idea belongs rather to the earliest stages of evolution and the formation of autotrophs from primitive heterotrophs. The following changes are distinguished [48] in the biochemical systems of animals during evolution: a) Specialization by quantitative or topographical changes; b) specialization by acquiring new component substances; c) specialization by the loss of some component substances; d) the use of biochemical substances from more primitive systems in new systems; e) specialization of old biochemical systems by introducing into them elements from other systems. For a solution of the problem of the path of change of substances and of biochemical processes during evolution it is especially necessary to study concrete chemical differences for substances of the different chemical groups in a phylogenetic series which has been set up on the basis of other criteria. The serological method has long been used [49, 50] to explain phylogenetic relations (the field of "serological taxonomy" [51]). This method permits only a demonstration of the degree of similarity or difference in the proteins of the organisms being compared. However, the method does not answer the question of the nature of the difference and should therefore be combined with a chemical study of the substances in the organisms being compared.

3. Biochemistry and Problems of the Factors in Evolution

Heredity and Variability. In connection with the practical demands of selection, there has been much study of heredity and variability of economically important biochemical traits [52] which has created the "biochemistry of variety" [53]. Along with this, heredity and variability have been studied by the content and structure of substances in the various chemical groups in connection with the explanation of the chemistry of their biosynthesis. This has produced theoretical investigations of the inheritance of flower color in plants and led to work on inheritance and variability of the ascomycetes Neurospora with respect to the ability to synthesize various substances [54]. We should particularly mention the studies on change in biochemical activity and chemical composition of organisms in interspecies variability, polyploidy, and sexual hybridization [52].

Michurin studies have established many important and pointed questions in this field and these have been treated by our biochemists and geneticists. In particular, studies have been conducted which indicate the inheritance of biochemical traits in the seeds of the descendants of vegetative hybrids [55]; the enzyme systems of Michurin varieties of apples have been studied [56], as has the enzyme activity of wheat, hard by heredity, and changed into soft by heredity [56], and the direction of enzymatic transformation of carbohydrates in summer wheat changed into the winter form [58].

The development of biochemical study of the exact nature of the mechanism of transmission of inherited properties (transmission of "genetic information") from one generation to another and the variability of this mechanism [18] (properly speaking, the biochemistry of heredity and variability concerning "biochemical genetics" [54, 59, 60]), has indicated the role of nucleic acids in the biosynthesis of proteins [61] and this has led to work on explaining the structure of nucleic acids and an explanation of the chemical mechanism of their self reproduction, the effect of various factors on these mechanisms, and the direction of changes in the processes of self reproduction of nucleic acids and proteins [18].

The study of the mechanisms of heredity and variability as biochemical processes has made our general ideas of the relation of heredity and variability to metabolism much more concrete, since no biochemical processes can occur in isolation from metabolism as a whole, with which it is connected through the available starting substances and energy.

Ideas have been introduced [62] of biochemical processes as "bundles" of biochemical reactions which proceed in many directions and occur because the numerous chemical reactions [63] and which form a mixture of substances (numerous examples of each group of substances in the organism [63]). A change in external conditions can strengthen or weaken different directions and even shift the main direction of a bundle of reactions. Especially in a bundle of reactions for the formation of proteins this can lead to creation of proteins with differing structures. Thus a change in the bundle of reactions lies at the basis of the process of variability of organisms under the influence of external conditions and this supplies the raw chemical materials for the evolutionary process, thus giving a basis for evolutionary development of organisms in different directions and presenting to the morphologist also a "bundle" of "infinitely varied phylogenetic branches" [64].

Natural Selection. Biochemistry has more and more found a very fine adaptation of chemical structure of substances and biochemical processes to participation in one or another function of the organism. It has shown [65] the use during evolution of very different physical

and chemical properties of substances of various groups for taking part in the construction of tissues and organs and carrying out various functions (thus, in certain transformations details of structure are suitable for some specific function). Darwin suggested [38] that "natural selection functions daily, hourly, in the whole world, rejecting the bad, retaining and strengthening the good, working unheard, unseen, everywhere and at all times, without leaving anything to chance;" this has acquired new significance and is newly illustrated by these biochemical facts. Biochemistry can only increase the amount of evidence of the power of the action of natural selection and in turn reject ideas which would devaluate its role in evolution. At the same time it shows the absence of absolute perfection in the biochemistry of the organism [66] analogous to the absence of absolute morphological and physiological perfection.

The statement of the fact of the multiplicity of uses of substances combined with the facts of the use of the same biochemical processes and enzyme systems in different functions leads to the conclusion that there is the complete use of the materials present in the organism for carrying out its functions [65]. This can be explained by the fact that minute changes in the structure of substances obviously occur much more often than major changes which lead to a new type of structure, and so the minute changes in the structure of substances of a given group, which do not modify the general structural plan, are more available in materials for the evolutionary process. The law of the complete use of materials present in the organism during evolution is confirmed also by the fact of the various uses of organs (the multi-functioning of organs) during evolution. This is an example of the fact that the study of biochemica relations can lead to conclusions of general biological significance.

4. Biochemistry and Problems of the Path (Direction) and Regularity of Evolution

The question of the direction of evolution from the biochemical angle is related to the question of the possible direction of transformation of the structures of substances in the main vital parts of the organism during evolution, with nucleic acids and proteins as the most important. These transformations cannot be by chance; they depend on the nature of the structure of the substances and the character of the bundle of biochemical reactions. Thus, for example, it has recently been shown that in proteins the quantitative ratio of individual amino acids is not arbitrary, but there are regularities in the arrangement of the amino acid radicals so that the number of amino acid combinations is less than is theoretically conceivable [14]. Within the limits of possible transformations of structure of substances in the organism, the evolutionary process may proceed in a large number of ways (besides, during evolution these"limits" may be changed as for example occurs in the formation of "unusual " structures in microorganisms).

In estimating the path of evolution of organisms from the biochemical angle, many authors start from the easily understood idea of an obligatory increase in complexity of the structure of substances during evolution as evidence of some definite direction of the chemical changes in evolution. For example, some authors [67-69] look for the formation of cyclic substances from aliphatic ones during evolution; others [70] look for an increasing molecular-weight of the substances. However, these expectations are not confirmed by the facts which relate to the stages of evolution from single-celled organisms to those with many cells or their further development. On the contrary, even in the earliest of these stages we find in the simplest organisms, such as the algae, substances with a complex structure in luding cyclic compounds (see the detailed discussion of this question [1]); as mentioned, a complex aggregation of substances occurs in the first organisms and is then used in the development of the more elaborate ones.

In fact, the direction of biochemical changes of the organisms during evolution is determined by changes in the external medium and the relations which are established within the organism (in its internal medium) and between the organism and the medium. Thus, with a definite direction of change of the medium there occurs as a consequence a corresponding direction of change in the biosynthesis of substances. For example, with the change from an aqueous to a terrestrial form of life there occurs a definite change in composition of the storage fats of the animal [71]. The direction of change in the composition of substances with a definite direction of change in external conditions of the medium is fully explained by Darwinism and nothing confirms the orthogenic idea of a predetermined direction of change of the organism during evolution.

EVOLUTIONARY DOCTRINE AND THE SOLUTION OF BIOCHEMICAL PROBLEMS

The doctrine of C. Darwin has given an evolutionary content to biological disciplines and has permitted their great development. At the time when Darwinism appeared, biochemistry was still not distinguished as a separate discipline. During the development of biochemistry the ideas of the theory of evolution did not affect it as strongly as they did many other branches of biology. Even the newest textbooks and reviews of biochemistry still do not include logical discussion of biochemical facts from an evolutionary viewpoint. In this respect much work remains to be done, but much has been accomplished; the comparative biochemistry of organisms has long been studied, and, as has been shown, has affected many sides of biochemistry. These comparative data are also fundamental for biochemists in explaining the problems of the doctrine of evolution which we have considered in the preceding discussion. These data should also be of help in solving problems of biochemistry. The comparative biochemical approach will be introduced into biochemistry in the course of time and will inevitably give notable results. For example, comparison of photosynthesis of higher green plants,

algae, and photosynthesizing bacteria has greatly widened our ideas of this process [41]; comparative biochemistry has disclosed important characteristics of nitrogen metabolism [72]. Ideas on the common origin of organisms with later divergence during evolution which explain the "unity among the differences" in biochemistry [29] permit us to direct biochemical investigations in an effective way. For example, on just this basis a way has been found recently for the study of the enzymology and mechanics of the chemistry of the contractile function [29].

We cannot now consider a biochemical process to be fully explained if the explanation does not include the change of this process in different organisms. An explanatio of the nature and amplitude of such changes gives a deeper idea of the nature of this biochemical process (for example, such a method has been used in the study of fat biosynthesis [42]).

Darwinism has enriched the comparative method of biology by its historical approach to comparative phenomena. "Wherever the historical method has been used in biology, it has thrown light on those areas which without it would have remained in shadow" [73]. In the development of biochemistry further introduction of the comparative (comparative-historical) method should be of great value.

SUMMARY

The materials presented in this paper have shown the reciprocal effects of biochemical facts give a new confirmation to the evolutionary doctrine of Darwin, and, in agreement with the facts of other branches of biology, permit solution of its problems. On the other hand, evolutionary doctrine permits us to treat the enormous amount of factual material of biochemistry and to direct further studies in the solution of specific problems of biochemistry, starting from ideas of the unity of evolutionary origin of organisms and the regularities of evolution. It is important to strengthen the bond between biochemistry and evolutionary doctrine; it is particularly important to develop evolutionary comparative biochemistry.

A. M. Goldovskii

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THE EVOLUTION OF PROTEINS AND THE EVOLUTION OF FLOWERING PLANTS

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Translated from Biokhimiya Vol. 25, No. 1, pp. 12-16, January-February, 1960

Original article submitted September 24, 1959

The book of Charles Darwin, "The Origin of Species" (1859) first gave a materialistic basis for the study of the evolution of organisms, a study which arose in ancient times, but before Darwin it was erected more on speculation than on factual material. From the time of Darwin physics and chemistry have made great strides forward, and on their biological side biochemistry and biophysics have arisen; this has unavoidably posed the question of whether along with the morphological evidences there has been equal evolution of protoplasm and its basis, proteins.

The work of Kossel [1] has showed that the protamins of different species of fish are not identical; the most specialized of them, salmine and clupeine , are obtained from the highly developed, phylogenetically young Clupeiformes. The protamines of the older Ganoideae (sturgeons) are markedly poorer in bases, the number of their amino acids is greater, and the specialization of the protein molecules is less. It is interestin that in ontogenesis the protamines are much less specialized than the histones.

The work of Osborn [2] with plant proteins showed the high specificity of the proteins (prolamines) of seeds of the family Gramineae. At the same time it showed the nonidentity of the prolamines of wheat, barley, rice, maize, etc.

These results of Kossel and Osborn, and also of other authors, have shown for both animals and plants a close relation of the characteristics of the proteins with the systematic position of the organisms, but do not indicate the nature of this relationship or its significance in the evolution of the organism. In addition, new facts from the field of comparative biochemistry have continued to attract attention. Danielson [3] has found that the group of globulins in the seeds of various cereals is not the same: in wheat he found α-globulin with a mol wt of 29,000 and γ-globulin with a mol wt of 210,000; in barley, the globulins α and β (mol wt 100,000), γ (mol wt 166,000) and δ (mol wt 300,000); in the seeds of Festuca rubra there is only α- and in the seeds of Festuca pratensis only γ-globulin (Danielson did not study the other proteins of these plants).

The data of Danielson on the globulins of legume seeds are much more interesting. In the great majority of cases he found in these seeds only two globulins: vicilin S_{20} 6.5-8.69, mol wt 186.000) and legumin(S_{20} 10.10-14.02, mol wt 331,000). In a number of species of Acacia there was either no legumin at all (A. alata, A. longifolia, A. penninervis, A. verticillata) or it was present in slight amounts (A. decipiens, A. farnesiana, A. saligna). This characteristic permitted the author to place the acacias in a subfamily.

The family of Leguminosae is at present classified into the subfamilies Caesalpinioideae, Minosoideae, and Papilionatae. The oldest Caesalpinioideae are chiefly woody plants with straight seeds, slightly zygomorphic flowers and 10 free stamens. The seeds of most of the species of this subfamily are very poor in nitrogenous compounds: the total nitrogen content varies from 1.08% (in Dialium corbisieri) to 2.8% (in Bauhinia esculenta). We found 2.81% in the seeds of Cercis siliquastrum L. and 2.8% in the seeds of Gleditschia triacanthos L. However, we found 5.90% total nitrogen in the seeds of Caesalpinia gilliesii Wall. We should mention that morphologically the Caesalpinia belong to the most highly organized form of the subfamily Caesalpinioideae.

Study of the protein complexes of the seeds of Cercis siliquastrum, one of the oldest forms of Caesalpinioideae, showed that there were no proteins in either water or salt extracts of these seeds, and only 0.5 M solutions of NaOH removed a protein which after many reprecipitations contained 14.92% total nitrogen and 0.54% nitrogen from free amino groups. In a second representative of this subfamily, Gleditschia triacanthos, water-soluble albumins were completely absent in the seeds and the remaining protein which was removed consisted of 52% globulins and 48% alkali-soluble proteins. Finally, in Caesalpinia gilliesii in the total amount of water-soluble protein extract we found 8.14% albumin, 78.32% globulins, and 13.54% alkali-soluble proteins.

Thus, for the more ancient Caesalpinioideae which we studied a low albumin content and a high content of alkali-soluble protein are characteristic.

In the seeds of representatives of the subfamily Mimosoideae the total nitrogen content, according to the literature, is on the average 4.20 ±0.19%. We found for Acacia dealbata Link. 4.44%, for Albizzia julibrissin Durazz., 4.63%, and for Lagonichium farctum (Banks et Sol) Bobr, 3.28%. Hence it follows that in the mimosas the nitrogen content in the seeds averages twice as much as in the Caesalpinioideae.

In the seeds of Albizzia julibrissin (this species belongs to the phylogenetically young group of Ingeae) we found in the total extracted proteins: 36.96% albumins, 13.28% of a globulin difficult to salt out, that is, with low molecular weight (vicilin), 40.72% of a high molecular weight, easy to salt out protein (legumin), and only 9.04% of an alkali-soluble protein. In the seeds of Lagonichium farctum, a species which belongs to the certainly older group of Adenanthereae, there was first a low content of total nitrogen, and second a predominance of legumin which contained 16.16% total nitrogen: there was 66.44% legumin, only 2.75% albumin, and 40.85% alkali soluble protein.

Albizzia julibrissin Durazz. resembles in the composition of its protein complex the species of the Acacia family which are characterized by a marked prevalence of vicilin over legumin. It can be shown that the representatives of the Acacia and Albizzia families belong to the younger forms of the minosas, and the Lagonichium family to the older, since, according to the observations of de Candoll [5], it resembles the Prosopis family and also the Acacia family, and also includes only one species which grows in the dry ravines of the eastern Mediterranean and Iran.

The oldest of the Papilionatae phylogenetically are the representatives of the Sophoreae (trees and shrubs with flowers which have 10 stamens). In nitrogen content (3.83%) the seeds of Sophora japonica L. resemble the seeds of the mimosas and the Caesalpinioideae, and in the form of their proteins they are similar to the latter. According to our data albumin is entirely lacking in them, and the content of the alkali-soluble proteins is 59.42% of all the extracted proteins. In a second example of the Sophoreae, Maackia amurensis Rupr. et Maxim., the total nitrogen content in the seeds (6.54%) is higher than in Sophora. We extracted from the seed meal 2.06% albumin, 12.09% globulin, and 16.17% alkali-soluble proteins, that is, the latter made up 52.3% of all the extracted proteins. Evidently on the basis of biochemistry, Sophoreae should stand at the very bottom of the genealogy of the Papilionatae.

Among the representatives of the Phaseoleae the mean content of nitrogen in the seeds of 27 species is 4.24%. We [6] analyzed the seeds of Phaseolus aureus Roxb., Ph. lunatus L., and Dolichos lablab L. The content of alkali-soluble protein in them was high and made up about one third of all the extracted protein:

Phaseolus aureus	34,23%
» *lunatus*	28,00%
Dolichos lablab	32,20%

Determination of the fractional composition of the proteins of the seeds of Ph. aureus and Dolichos lablab gave the following results (%):

	Phaseolus aureus	Dolichos lablab
Albumin	3.94	12.40
Vicilin	54.80	} 55.33
Legumin	7.03	
Alkali-soluble protein	34.23	32.20

From the sedimentation curves in the work of Danielson we calculated the ratio vicilin/legumin and found:

Dolichos lablab	91 : 41
Phaseolus coccineus	39 : 15
» *nanus*	88 : 12
» *vulgaris*	84 : 28

The results of fractionation of Ph. aureus give a ratio of the same order, 55 : 7.

Galegoideae in number of species, (according to the "Flora of the USSR" [7], they include 1218 species out of 1805 species of the Leguminosae) are certainly far advanced on the path of evolution of the Papilionatae. We have determined the total nitrogen content in the seeds of 16 species. The seeds of the most primitive Galegoideae, Amorpha fruticosa L. contain only 4.65%, and among the most advanced in evolution astragalus, the average nitrogen content (from data of the determination in 10 species) is 8.49%, and in Astragalus Sieversianus Pall. it is 9.00%.

In Galega orientalis 59.19% of all the extracted protein in alkali-soluble protein, 19.83%is globulin, and 20.98% is albumin. Washing and drying the alkali soluble protein results in loss of 2.42% of nitrogenous substances based on weight of starting meal, and as a result of this treatment of the easily salted out globulin, the loss is 6.43% of the weight of meal. In Robinia pseudoacacia L. the protein removed in our laboratory was separated into water soluble (≈20%), salt soluble (≈70%) and alkali soluble (≈10%). When the salt used for salting out, $(NH_4)_2SO_4$, was concentrated from 26 to 60%, 39% of the soluble protein was salted out, and the remaining 61% precipitated only at concentrations of 81-87% of full saturation. In other words, in Robinia pseudoacacia vicilin predominated in the seeds. On the other hand, in Caragana arborescens Lam. we found predominance of legumin over vicilin (ratio of vicilin/legumin was 43 : 57) [4].

In Astragalus cicer L., 10.92% of the weight of the seeds was alkali-soluble protein, 6.99% albumin, and 1.76% globulin difficult to salt out. In addition, when we washed and dried the alkali-soluble protein, the filtrate contained 1.22% of the weight of the seed meal nitrogen, which corresponds to 7.64% of conventional protein (1.22 × 6.25); part of the nitrogenous substance was lost in washing and drying the globulins difficult to salt out (0.94% conventional protein), 1.22% nitrogen remained in the meal after extraction by all the solvents (1.22 × 6.25 = 7.64) and, finally, the low molecular weight substances which dialyzed through cellophane were 4.90% (0.78 × 6.25). Thus, in these plants globulin is almost absent, this typical "vegetable protein"; and the amount of albumin, the existence of which in plants has still been denied recently, makes up 24.74% of all the extracted protein.

Finally, in the recently studied example of the Galegoideae, Wistaria sinensis (Sims) D. S. all three fractions of the extracted protein are almost the same: albumin makes up 31.92%, globulin 34.10%, and alkali-soluble protein 33.98%.

These results show that for the examples of the Galegoideae studied there is a characteristic high content of

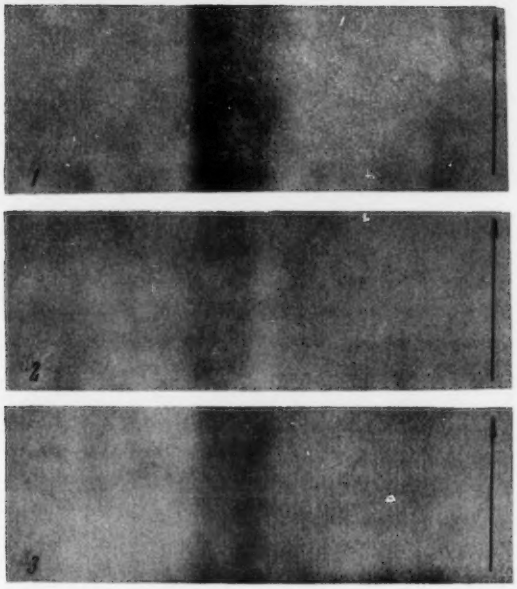

Fig. 1. Paper electrophoresis of extracts from seeds of Onobrychis arenaria (1), Cercis siliquastrum (2), and Lathyrus aureus (3). Phosphate buffer 0.2 M, pH 7.0; μ1.2, 22 hours, 125 v, 19 ma. Arrow indicates line of introducing sample

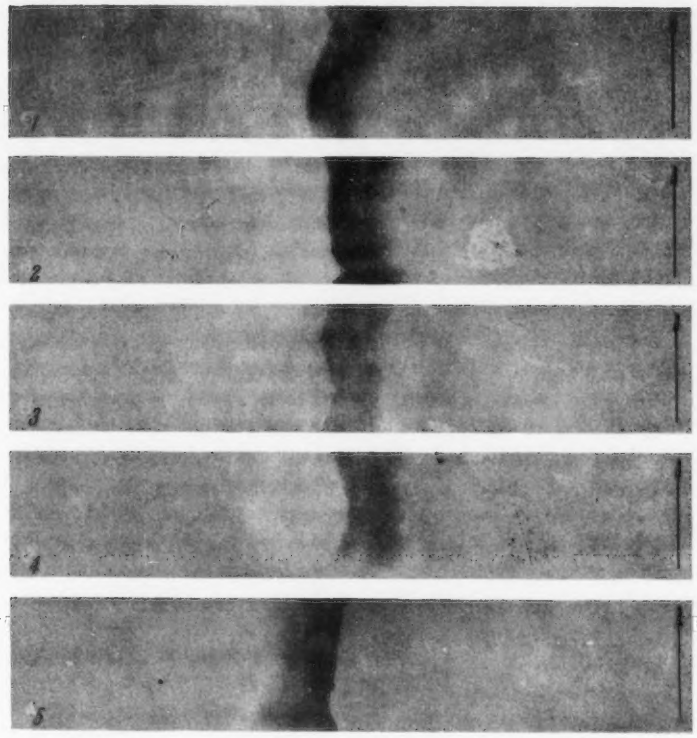

Fig. 2. Paper electrophoresis of extracts from seeds of Onobrychis tanaitica (1), O. Ruprechti (2), Lathyrus miniatus (3), Pisum thebaicum (4), P. sativum (5). Duration 44 hours. Other conditions the same as in Fig. 1.

alkali-soluble protein (in the most ancient form, Galega orientalis Lam. almost 60% of all the extracted protein). The albumin content is high; as to the globulin, in most of the Galegoideae it is absent, and only in Robinia pseudoacacia and Caragana arborescens is it contained in quantity.

Among the Hedysaroideae with their segmented beans there are certainly forms of an archaic character, like Arachis hypogaea L. which de Candoll [5] places among the Caesalpinioideae. since the seeds are not bent, as in all the Papilionatae, but straight, as in the Caesalpinioideae According to Danielson, in arachis seeds there is three times as much legumin as vicilin.

In Hedysarum alpinum L. we find the alkali-soluble protein 10.82% of the weight of the meal and globulin 9.31% washing with acetone and drying removes 2.02%; the unremoved nitrogenous substances are 7.24%, the low molecular weight dializing substances 2.04%. In Onybrychis sibirica Turcz. the seeds contain 9.86% nitrogen. Isolated alkali-soluble protein is 2.07%, albumin 4.13%, globulin 10.66%. In Onybrychis tanaitica Spreng. with a nitrogen content in the seeds of 10.38% we isolated alkali-soluble protein to the extent of 3.28% of the weight of seed meal, albumin, 4.09%, and globulin, 13.50%. We would mention the characteristic that the easily salted out globulin is both species of Onobrychis showed a high nitrogen content: 20.38% in O. tanaitica and 20.59% in O. sibirica.

Systematics relate the families Hedysarum and Onobrychis to the same order of Euhedysareae. Among these the nature of the proteins is very different, and it can be said that Hedysarum alpinum is phylogenetically much older than either form of sainfoil, that is, the independent development of the family is shown here still more sharply than in the Galegoideae (in the seeds of which we did not find a prevalence of alkali-soluble proteins over all the remaining proteins).

For the Vicioideae we did not carry out preparative fractionation of the seed proteins, but only compared their electrophoretograms (Figs. 1 and 2)(apparatus ÉFA-1; phosphate buffer, pH 7, and μ = 1.2, voltage 120 v, current strength 0.5 ma/cm; stain, bromophenol blue). We studied acetone preparations from meal of the seeds of Lathyrus aureus, L. miniatus, Pisum thebaicum, and P. sativum. The exposure for L. aureus was 22 hours, for the others, 44 hours. At an exposure of 22 hours we also submitted to electrophoresis the proteins of the seeds of Onobrychis arenaria and Cercis siliquastrum, and at an exposure of 44 hours, proteins of O. Ruprechti and O. tanaitica.

There are differences in the protein composition of the seeds of vetch and peas. In the latter the more mobile vicilin predominates, and the content of legumin is very low. In vetch (L. aureus) the legumin band is clearer than the band of vicilin, and in L. miniatus both bands are strongly developed. The electrophoretograms for the examples of Onobrychis resemble those for the species of Pisum. Thus, in the phylogenetically older species of Lathyrus there is obvious prevalence of the less mobile globulin of the legumin type, which is confirmed by our analysis of the sedimentation curves of Danielson, in which the ratio of areas of vicilin and legumin is:

Lathyrus clymenum	76 : 112
» *odoratus*	16 : 63
» *sativus*	65 : 216
» *silvestris*	76 : 112

For Pisum sativum this ratio is 140 : 105 [6].

SUMMARY

In seeds of phylogenetically older, primitive groups of legumes the alkali-soluble proteins predominate; while the content of albumins and vicilin is low. Young, progressively evolving groups of legumes, on the other hand, are characterized by the predominance of vicilin over legumin, a higher content of albumins, and a low content of alkali-soluble proteins.

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CHANGES IN THE NUMBER OF PEPTIDE BONDS IN ISOLATED PROTEINS*

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Translated from Biokhimiya Vol. 25, No. 1, pp. 17-23, January-February, 1960

Original article submitted July 18, 1958

A series of studies has established [1-6] that when isolated proteins react with their structural units – free amino acids and peptides – these are transferred into the protein molecule, and there is formation, between the amino acids and other constituent parts of the protein, of peptide bonds and other bonds characteristic of the protein molecule.

It is very important to explain whether after such a transfer of the structural units of the protein into its molecule there is merely renewal of the molecule without change in the total number of peptide bonds, or whether the addition of the amino acids and peptides to the isolated protein under definite conditions is accompanied by increase in the total number of peptide bonds and amino acid radicals in the protein.

Increase in the amount of protein during its reaction with free amino acids has been observed in isolated biological structures such as: sections of pigeon liver [7], sections of diaphragm [8], liver microsomes [9], chloroplasts of plant cells [10], microbial protoplast [11], and in the ribonucleoprotein portion of plant microsomes [12].

In the present work we have studied the change in number of peptide bonds of myosin depending on the presence of its hydrolyzate, ATP, and also nucleic acids. Along with this, under identical conditions, we have studied the process of uptake in isolated myosin of the products of hydrolysis of this protein first labeled with radioactive amino acids. Also, for comparison of the course of these processes is a system of protein-amino acids, and the same in a complex biological system, and analogous investigation has been carried out with isolated liver nuclei.

METHODS

In the experiments with isolated myosin we used three times reprecipitated myosin isolated from rabbit muscle, and also a labeled myosin hydrolyzate. The labeled hydrolyzate was prepared by boiling myosin for 40 hours with 6 N HCl. This myosin had first been labeled in vivo with radioactive amino acids, tyrosine-C^{14} and glycine-C^{14}; the radioactivity of the resulting hydrolyzate was 10,000 counts/min/ml. 1 ml of hydrolyzate contained 5 mg N; according to our observations and the data of Marecek [13] peptides were absent from the hydrolyzate obtained in this way.

The number of peptide bonds in the protein sample was determined by several methods; especially by the method of Lowry [14] and by the method of Waddell and Hill [15]. The latter method is based on the determination of the absorption values of the protein solution in the limits λ 225-215 mμ. Absorption in this range guarantees the presence of peptide bonds. For determination of the number of peptide bonds by the method of ultraviolet spectrophotometry we took samples which contained in 1 ml of diluted myosin solution, 5 ml of Ringer-phosphate Krebs buffer, pH 7.5; to part of the sample also we added the myosin hydrolyzate which contained 0.1-0.2 mg N. To another part of the sample we added along with the hydrolyzate 0.5 mg of ATP/ml of sample.

After addition of the hydrolyzate the experimental samples were incubated 30 min at 37°; after this the protein was precipitated by trichloroacetic acid at a final concentration of 5%. The control samples (I) were incubated without hydrolyzate which was added before precipitation of the protein. The control samples (II) were used without incubation and the protein in them was precipitated at once.

The samples after addition of trichloroacetic acid were centrifuged to the formation of a dense protein precipitate and then the supernatant liquid was carefully removed and the precipitate was dissolved in 1 N KOH. This gave a clear solution which was subjected to photometry in the Beckman DU spectrophotometer at λ 225-215 mμ. In some of the experiments trichloroacetic acid was not added to the sample, and the volume was brought to 100 ml with 0.25 M KCl and the diluted solution was subjected to photometry.

In the experiments on the study of the peptide bonds in the presence of nucleic acids and the method of ultraviolet spectrophotometry with measurement of the optical density after addition of the compounds was replaced by the method of Lowry [14]. For the determination of the number of peptide bonds by this method we usually took 1 ml of myosin in the experimental and control samples, diluted it in 0.5 M KCl, 2 ml of carbonate-phosphate buffer pH 8.6, and 1.5 ml of hydrolyzate which contained 0.1-0.02 mg N per ml. The experimental samples were incubated for 30 min at 37°. Part of the control samples was

* From the Editor. The Editor publishes this paper, yielding to the urgent request of the authors. The Editor accepts no responsibility for the material published here or the conclusions drawn.

not incubated, another part was incubated without the hydrolyzate which was added to it after incubation. Then we took 1 ml of solution from the experimental and control samples and added reagents as described by Lowry to develop the color reaction. Measurement of the intensity of the color was carried out in a Hilger electrophotocolorimeter at λ 755 mμ. Calculations were made on a calibration curve and the data were expressed in equivalent amounts of protein.

For study of the process of uptake of radioactive hydrolyzate in isolated myosin we used the same myosin preparation and hydrolyzate. Samples in this series of experiments consisted of 30-20 mg of protein (myosin); 10 ml of phosphate buffer, pH 7.5, and 1 ml of hydrolyzate which contained 5 mg of N, obtained by hydrolysis of myosin. The protein for calculating the radioactivity was prepared by the method described earlier [1]. The radioactivity in the protein was measured with an end counter. The square of the deviation did not exceed ±5%.

For carrying out the experiments with isolated nuclei they were separated from rabbit liver by the method of Hogeboom et al.[16]. The separated nuclei preparation contained less than 1% of undestroyed cells and mitochondria. In the experiments on determination of the value of uptake of protein hydrolysis products in the nuclear proteins we used a liver hydrolyzate labeled with glycine-C^{14} or tyrosine-C^{14}. The samples consisted of 0.3-0.5 ml of nuclear preparation which contained 1-1.5 mg of protein and 1 ml of labeled hydrolyzate with a radioactivity of 10^6 counts/min which contained 1.10 mg N. The medium for incubation was a solution of 0.25 M sucrose and 0.0013 M $CaCl_2$ in a Krebs buffer; the total volume was 5 ml. The experimental samples were incubated 30 min at 37°, after which 25% trichloroacetic acid was added to a final concentration of 5%; the samples were centrifuged, the supernatant liquid was drawn off, and the protein precipitate was analyzed by the Kjeldahl method; the amount of $(NH_4)_2SO_4$ formed was determined in a Conway vessel. The differences between parallel experiments did not exceed 3%.

For each experimental sample we used two controls. Control I differed from the experimental sample in that hydrolyzate was not added to it. Hydrolyzate was added to control II directly after incubation before adding trichloroacetic acid to the sample. All the control samples after precipitation by trichloroacetic acid were given the same treatment as the experimental samples.

Parallel with these we ran samples to determine the radioactivity in the nuclear proteins after their incubation with labeled hydrolyzate. After the proteins had been precipitated by trichloroacetic acid we added to them 10-25 mg of nonradioactive liver protein as a "support." All the protein samples were stirred and redissolved in 3% alkali and after repeated precipitation by trichloroacetic acid were treated as described in [1]. The radioactivity was calculated per 5 mg of dry protein, using an end counter; we corrected for the dilution produced by adding the ballast liver protein.

RESULTS

As Table 1 shows, the data obtained by the method of ultraviolet spectrophotometry indicate that when myosin is incubated in the presence of the hydrolyzate there is an increase in optical density. This increase reflects an increase in the number of peptide bonds which corresponds to an increase in the total number of amino acid radicals in the protein. In the experiments with addition of hydrolyzate and ATP (Table 1) the increase in peptide bonds is found only in a few of the samples. In the great majority of the experiments there was a fall in total number of peptide bonds.

In Table 2 we give data obtained by the Lowry method on the change in number of peptide bonds in myosin incubated in a medium with a full selection of amino acids in the presence of DNA and without it. In the great majority of the experiments (Table 2) we found in the protein under investigation after incubation in the presence of a full selection of amino acids both with and without DNA a larger number of peptide bonds than in the controls, which differed from the experimental samples in that the amino acids were added to the medium only at the end of incubation of the myosin. Incubation of myosin in the presence of amino acids and DNA in many experiments was accompanied by a more marked increase in peptide bonds than in the absence of DNA.

Thus, the material which we have obtained shows that when there is reaction in the system protein-amino acids of myosin which are its structural units, they are utilized and there is increase in the total number of peptide bonds.

Parallel with the determination of total number of peptide bonds of myosin when it is incubated with the hydrolyzate we considered it important to study the transfer of the products of myosin hydrolysis into the composition of its molecule, using labeled hydrolyzate. We carried out this investigation to show the relation between the increased number of peptide bonds in myosin and the amino acids entering its composition from the surrounding medium under these incubation conditions. When labeled hydrolyzate was added to isolated myosin after it had been prepared from the same protein, there was an uptake of free amino acids by the myosin (Table 3). The amount of this uptake varied from 2.12% to 4.1% N of the hydrolyzate with respect to N in the initial amount of protein of the sample. This value was of the same order as in our earlier experiments on the increase in number of peptide bonds but the absolute value was somewhat lower. The reason for this difference is evidently the different method of treating the sample.

However, spontaneous quantitative changes in the isolated protein do not come only from the increased amount of peptide bonds and amino acid radicals. In the case of incubation of isolated proteins, especially myosin,

TABLE 1. Change in Optical Density During Reaction of Myosin with Free Amino Acids (time of experiment 30 min, temperature 37°)

Myosin + hydrolyzate			Myosin+ hydrolyzate + ATP		
experimental	control	% change	experimental	control	% change
0,805	0,782	+3,5	0,782	0,790	−0,9
0,849	0,725	+17,1	0,750	0,690	+8,0
0,716	0,679	+5,6	0,720	0,680	+5,9
0,758	0,694	+9,2	0,737	0,780	−5,5
0,549	0,625	−12,0	0,578	0,640	−9,7
0,811	0,698	+16,1	0,720	0,749	−3,8
0,604	0,648	−6,7	0,544	0,556	−2,0
0,817	0,700	+11,8	0,715	0,749	−4,5
0,447	0,420	+10,6	0,460	0,503	−8,5

TABLE 2. Change in Number of Peptide Bonds in the Reaction of Myosin with Free Amino Acids (number of peptide bonds, equivalent to a γ of protein. Numbers in parentheses, % change)

Without DNA		In the presence of DNA	
control	experimental	control	experimental
217,0	220,0 (+2,6±0,75)	146,0	155,0 (+6,3±0,6)
159,0	163,0 (+2,2±0,36)	143,0	151,0 (+5,6±0,46)
151,0	154,0 (+2,0±0,46)	148,5	164,0 (+10,4±0,94)
146,0	156,5 (+7,2±0,26)	99,0	105,0 (+6,1±0,55)
146,0	153,5 (+5,1±0,24)	99,0	100,0 (+1,0±0,13)
99,5	99,5 (0,0)	57,0	65,0 (+14,0±1,3)
61,5	65,0 (+5,6±0,31)	43,0	50,5 (+17,4±0,9)
61,5	65,0 (+5,6±0,31)	43,0	48,0 (+11,6±0,9)
92,0	84,5 (−8,1±1,70)	223,0	216,5 (−2,9±0,16)
134,0	137,0 (+2,2±0,0)	119,5	127,5 (+6,7±0,57)
124,5	130,5 (+5,0±0,63)	113,0	131,0 (+15,9±0,6)
212,5	212,5 (0,0)	111,0	109,0 (−1,4±0,0)
		113,0	123,0 (+8,0±1,1)
		93,0	100,0 (+7,5±0,57)

TABLE 3. Uptake of Myosin of the Hydrolyzate Labeled with Glycine-C^{14} and Tyrosine-C^{14} (time of experiment 30 min, temperature 37°)

Myosin incubated with radioactive hydrolyzate		
counts/min · 5 mg protein		N taken up from hydrolyzate with respect to N of protein sample, %
54		3.37
65		4.05
58		3.61
45		2.80
46		2.86
52		3.74
66		4.11
34		2.12
Av.	52.5	3.32

in the absence of a complete selection of amino acids we found by the use of the Lowry method (Table 4) a decrease in the number of peptide bonds. This leads us to believe that in the system protein-amino acids there are two opposing processes: a transfer of the structural units of the protein from the medium into the molecule, and a removal of constituent parts of the protein molecule into the medium; the presence in the medium of a full selection of amino acids will then determine the prevalence of increase in peptide bonds over their splitting. This leads us to think that the splitting of the protein molecule of myosin occurs not only as the result of the action of specific enzymes on it, but also by a direct mechanism belonging to the protein molecule itself.

In the experiments with isolated liver cell nuclei run in parallel with the studies of entry of free amino acids into the protein in the uptake of radioactive products from the protein hydrolyzate, we used still another method for studying this effect, the method of determining the change in total amount of protein by protein nitrogen [10].

TABLE 4. Change in Number of Peptide Bonds After Incubation of Myosin in the Absence of Hydrolyzate

Experiment No.	No. of peptide bonds equivalent to 1γ protein		Change, %	Experiment No.	No. of peptide bonds equivalent to 1γ protein		Change, %
	before incubation	after incubation			before incubation	after incubation	
1	201,0	197,5	—1,70	5	89,0	85,0	—4,40
2	172,5	165,6	—4,10	6	167,5	160,0	—4,50
3	177,0	171,5	—3,10	7	171,1	160,0	—6,40
4	185,0	183,5	—0,81	8	94,5	87,5	—7,40

TABLE 5. Increase in Nitrogen of the Trichloroacetic Acid Precipitate after Incubation of the Nuclear Fraction of the Liver (N in γ/sample; time of experiment 30 min; temperature 37°)

Nuclei without hydrolyzate (A)	Nuclei + hydrolyzate, added after incubation (B)	$\frac{B-A}{A}$, %	Nuclei + hydrolyzate, added before incubation (C)	$\frac{C-B}{B}$, % increase
351,4	360,9	+2,7	376,1	4,2
76,2	74,6	—2,1	86,8	16,4
156,8	151,4	—3,4	164,1	8,3
94,1	95,9	+1,9	109,5	14,2
110,9	116,3	+4,8	129,1	11,0
159,9	167,5	+4,7	183,4	9,5
204,8	201,6	—1,5	221,9	10,0
205,8	200,2	—2,7	243,6	21,6
179,9	188,6	+4,8	202,6	7,4
Average		+1,02		11,4±1,72

The data on increase of trichloroacetic acid precipitate show (Table 5) that when the liver nuclei are incubated in the presence of the protein hydrolyzate there is increase in the nitrogen of the trichloroacetic acid precipitate on the average by 11.4 ± 1.72%, while in the control samples the change is 1.02%. Thus, the size of the increase in nitrogen in the trichloroacetic acid precipitate in all the experimental samples was greatly in excess over the value of this increase in the control samples. This increase in the experimental samples exceeds the value of autolysis which in separate experiments varied from 2.5 to 7.5%. Hence the difference obtained in the values for nitrogen of the trichloroacetic acid precipitate between the experimental and control samples cannot depend solely on inhibition of splitting of the nuclear proteins by the hydrolyzate during incubation but depends on the transfer of free amino acids of the medium into the protein molecules of the isolated nuclei; this is also shown by data obtained in experiments with the uptake of radioactive hydrolyzate in the proteins of the nuclei. It is characteristics that the addition of enzyme poisons such as 2,4-dinitrophenols or hydroxylamine in concentrations of 10^{-3} M to the experimental samples decreased the increase in nitrogen of the trichloroacetic acid precipitate by 90%.

DISCUSSION

The results given in this paper for experiments with isolated myosin, obtained by the method of Lowry, by labeled atoms, and by ultraviolet spectrophotometry, have shown that when myosin reacts with free amino acids of the medium these are transferred into the composition of the protein, as a result of which there is an increase in the number of peptide bonds and amino acid radicals of the protein. These results indicate that under definite conditions, especially when myosin is incubated with the mediun which contains a full assortment of amino acids, the uptake of labeled amino acids reflects not only the renewal of the protein, but an increase in the number of its constituent parts.

When myosin is incubated in a buffer solution in the absence of hydrolyzate, we find a spontaneous decrease in the number of peptide bonds in the sample. This effect indicates that isolated myosin can undergo a process of autodecomposition similar to the process which it undergoes of taking up free amino acids from the medium. When ATP is added to myosin under the conditions of our experiment there is an increase in its autolysis, as was found in the experiments of Simpson [17] with the addition of ATP to surviving liver slices.

The study of the reaction of isolated liver nuclei with free amino acids of the hydrolyzate by the Kjeldahl method and the use of isotopic indicators gave analogous results. The uptake of free amino acids of the medium in the composition of the nuclear proteins was accompanied by increase in the amount of amino acid radicals of the protein.

The increase which we found in total number of amino acid radicals of the protein as a result of uptake of the free amino acids of the medium can be interpreted in different ways. The increased number of peptide bonds in the protein or the amount of protein nitrogen until recently has been considered to be a single phenomenon, formation of new protein; however, with accumulation of data on the process of biosynthesis of proteins in isolated systems it has become evident that this general phenomenon can be the result of several processes which differ from each other in chemical and biological respects.

The data of Peters and Anfinsen [7], Peters [18], Straub [19], Sthamann [20] and others permits the possibility of assuming that in an understanding of increase in total amount of protein we must include: Formation of new protein molecules with a special configuration and packing of the peptide chains which are characteristic of the existing form of the protein; synthesis of new protein molecules without specific packing and combination of the separate fragments of the peptide chains or amino acid radicals with different parts of the protein molecule.

All these effects in the synthesis of proteins lead to an increase in the total amount of nitrogen in the trichloroacetic acid precipitate, the number of peptide bonds and of amino acid radicals of the protein. They cannot be differentiated by methods based on calculations by the above indexes or by biological effects. As is evident from the work of Straub [17] for an increase in enzymatic activity of amylase it is not necessary that the whole protein molecule be constructed anew; it is enough that only two amino acids take part in the reconstruction of the protein molecule. Similarly, according to the work of Sthamann, even artificial growth of peptide chains can change the immunobiological properties of a protein though this does not cause new formation of the whole protein molecule.

Evidently, all these effects connected with the increase in mass of the protein are significant, but have differing biological significance. These investigations have not shown which of the above mentioned possible effects causing increase of peptide bonds occur in our experiments with isolated myosin or isolated liver nuclei. However, the sameness of the conditions under which increase in peptide bonds occurs in isolated myosin and in isolated nuclei, and the similarity in the order of magnitude of this increase, indicate the presence of some similarity in the production of these processes in both materials studied.

SUMMARY

We have shown that when myosin reacts with a complete assortment of amino acids there is a transfer of the free amino acids of the medium into the composition of the myosin, accompanied by an increase in the total number of peptide bonds of the protein and an increase in its amino acids radicals. The intensity of this process increases in the presence of deoxyribonucleic acid.

We have found that incubation of isolated myosin in a buffer solution in the absence of amino acids is accompanied by decrease in the total number of peptide bonds.

We have established that in the reaction of isolated liver nuclei with free amino acids of liver protein hydrolyzate there is a transfer of free amino acids of the medium into the composition of the isolated nuclei and an increase in amount of amino acid radicals in their protein.

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THE DISTRIBUTION OF PHOSPHATIDES IN THE ORGANS OF SALMON

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Translated from Biokhimiya Vol. 25, No. 1, pp. 24-27, January-February, 1960

Original article submitted September 23, 1958

Phosphatides are widely distributed in animal and plant tissues, but very little is known as to their role in the organism. One of the characteristics of phosphatide distribution is their high content in reproductive substances. Thus, lecithin is largely found in egg yolk, where it was first discovered; phosphatides are found in considerable quantity in role [1], and choline in animal sperm [2, 3].

An increased amount of lipides, including phosphatides, is found in the blood of chikens during the period of intensive egg laying [4] and also in the blood of female turtles at the time of activation of ovarian function [5].

Wada [6] observed an increase content of lecithin in sardines immediately before spawning. An important, but little studied question relates to the relation between the content of glycerol phosphatides and neutral fats. The similarity in structure suggests their similarity in origin or in their transformations, and experimental data confirm this. Kennedy and co-workers [7-9] consider that when diglycerides react with acetyl coenzyme A they are transformed into triglycerides, and when they react with cytidine diphosphocholine or with cytidine diphosphoethanolamine they form lecithin or ethanol amine phosphatides.

In the present work we have determined the total amount of lipids, the content of lipid phosphorus, and of choline in some organs and tissues of the salmon Salmo salar Linné.

METHODS

We studied six fish (3 male and 3 female) caught during the spawning period in the last part of October. The first two pairs of salmon were caught in the Neva and the third pair in the Daugava; the individuals of each pair were analyzed at the same time. The male and female of the first pair had not yet had time to spawn, but the males of the second and third pair had already discharged part of their semen (they discharged it in small portions on several occasions). The female and male of the first pair weighed 6-8 kg, and the males of the second and third pairs weighed 3.5-4 kg.

In the freshly caught salmon the aorta was cut at the heart and the blood was collected in a weighed container. Then the fish was decapitated and the internal organs removed. Samples of muscle were taken by cutting crosswise on one half of the body from the spine to the stomach. The samples were weighed and coarsely ground. For collecting the roe, one ovary was freed from large blood vessels and heavy tissues, ground up, and the sample of roe taken along with the thin walls of the ovary. The milt was also weighed along with the walls of the testes. The intestines (from stomach to cloaca) were freed from their contents, washed with water, dried with filter paper, and weighed. The samples were placed in a jar filled with alcohol and kept until analysis in a refrigerator at about 0°. From the moment of removal of the fish from the water to the addition of alcohol to the prepared sample was not more than an hour.

For extraction of the lipids, the material along with the alcohol was transferred to a flask and boiled for three hours under reflux. Then the alcohol extract was filtered, the material was ground with glass, placed in a paper pack, and twice more extracted with fresh portions of alcohol (6 and 3 hours). After removal of the alcohol, the material was extracted with chloroform in a Soxhlet apparatus for 6 hours. The combined alcohol extracts were evaporated almost to dryness under diminished pressure with passage of carbon dioxide through a capillary and addition of the chloroform extract to the residue. The resulting cloudy solution was washed in a separatory funnel with water, the chloroform layer was poured into a calibrated flask, and the water layer in turn was washed with small amounts of chloroform which were poured into the calibrated flask. The cloudiness which usually appeared was removed by addition of a small amount of alcohol. Chloroform was added to the mark, and definite portions were taken for analysis.

For determination of the total amount of lipids, the chloroform was distilled off in a weighed flask, the residue was dried in a vacuum drying apparatus, and weighed. P was determined by the Fiske-Subbarow method, modified by Horecker and co-workers [10].

Besides the total P in some samples we also determined the acid soluble P after hydrolysis of the lipids with 1 N KOH for 18 hours at 37°. Schmidt and co-workers [11] consider that this acid soluble P is the P of monoaminophosphatides which can be hydrolyzed under these conditions, and the difference between the total P of the lipids and the acid soluble P represents the P of the diaminophosphatides which are not hydrolyzed under these mild conditions. On this basis a micromethod has been worked out for the quantitative determination of different phosphatide fractions [12], but in

TABLE 1. Lipid Content in Salmon Organs and Tissues

Sample studied	Total lipid content, %	Lipid, P, mg %	Phosphatide P × 25, mg %	Phosphatide, in % of the lipids	Choline, mg %	Mole ratio of choline to phosphatides
First Pair						
Muscle of female	8,30	42,6	1065	12,8	72,3	0,43
Muscle of male	4,04	29	725	17,9	63	0,56
Roe	8,86	150	3750	42,3	478	0,82
Milt	2,76	55,4	1385	50,2	85,9	0,40
Liver of female	4,24	93	2325	55	161,8	0,45
Liver of male	3,46	86	2150	62	171,8	0,47
Second Pair						
Muscle of female	6,27	35,5	887	14,1	72	0,52
Muscle of male	6,60	30,0	750	11,4	58	0,50
Roe	8,65	144,5	3612	41,8	430	0,76
Milt*	1,71	39	975	57	34	0,22
Liver of female	4,42	105	2625	59,4	236	0,58
Liver of male	5,64	104	2600	46,1	237	0,58
Intestines of female	7,02	54	1350	19,3	109	0,52
Intestines of male	8,24	53	1325	16,1	115	0,55
Blood of female	1,61	33	825	51,3	86	0,67
Blood of male	1,54	28,8	745	41,9	81	0,70
Third Pair						
Muscle of female	3,68	30	750	20,4	75,3	0,64
Muscle of male	3,89	30	750	19,3	71,6	0,61

*The absolute content of lipids, P and chlorine in the milt was not determined accurately since the milt was diluted with water; however, the figures of the corresponding columns (underlined) allow us to estimate the ratio between the separate components.

our study the difference between acid soluble and total P of the lipids was very small and did not exceed the limits of error of the determination. Choline was determined by the method which we described previously [1]. The results of the analyses are given in Table 1.

In some of the samples were determined the content of P in the water layer which remained after washing the chloroform extract. Calculation of the content of this water soluble P gave the following (in mg%): roe 12, milt 7.8, male liver 9.5, female liver 11.5.

RESULTS

From the study of the organs and tissues the first place in lipid content was occupied by roe; then followed intestines and muscle; there was less lipid in the liver. In phosphatides, the first place was also held by the roe; then followed liver, milt, intestines with pyloric appendages, muscle, and blood. In Table 2 we give literature data on phosphatide content in the corresponding organs and tissues of mammals which shows that the organs and tissues of the salmon do not differ much in phosphatide content from a mammalian organs and tissues.

In the phosphatide content is expressed in percent of the total amount of lipids, the organs and tissues studied can be divided into two groups: The first group contains liver, roe, milt, and blood, in which the phosphatides make up 40-60% of the total amount of lipids; and the second contains intestines with the pyloric appendages and muscle; the content of phosphatides in these organs does not exceed 20% of the total quantity of lipids. We do not have enough material for an estimate of the relation between phosphatide content and total quantity of lipid lipids in similar organs and tissues, but we have the impression that in muscle the quantity of phosphatides varies comparatively slightly and is not related to the total quantity of lipids.

The highest quantity of choline and ratio of choline phosphatides to total quantity of phosphatides are also found in the roe. We should remark that the ratio of choline phosphatides to total phosphatides in the organs and tissues of the salmon vary within the same limits as in the organs and tissues of mammals, except for the testes. We found a comparatively small amount of choline in the milt, while there are reports [2, 3, 13] of a high choline content in the testes and sperm. This difference may be due to the pres-

TABLE 2. Content of Phosphatides and Choline and Mole Ratio of Choline Phosphatides to Their Total Amount in Organs and Tissues of Mammals

Animal	Material studied	Phosphatide content, mg%	Choline content, mg, %	Mole ratio, choline to total amt. of phosphatides	Authors
Rat	Liver	2960—3080	271—295	0,56—0,64	K. Artom [13]
	Muscle	938—1000	69—80	0,45—0,51	
	Testis	1247	102	0,52	
Rat	Sperm	—	514	—	J. Fletcher et al. [2]
	Liver	—	260	—	
	Small intestine	—	142	—	
	Blood (in starvation)	—	22	—	
Cow	Liver	—	270	—	
	Muscle	—	76	—	
Dog	Liver	—	230	—	
	Blood	—	34	—	
Cod	Muscle	—	78	—	
Cow	Liver	—	—	0,60	M. Kaucher et al. [14]
	Muscle	—	—	0,63	
	Intestine	—	—	0,73	
Dog	Liver	—	—	0,55	McKibbin and Taylor [15]
	Muscle	—	—	0,48	
	Intestine	—	—	0,51	

ence in the semen of a large amount of choline-containing products from the splitting of lecithin and of free choline, which has been mentioned in the literature [16]. We did not consider these substances.

We did not find much difference between the organs and tissues of males and females as to their content of lipids, phosphatides, and choline, except for the roe and milt.

SUMMARY

We have determined the content of lipids, lipoid phosphorus, and choline in the organs and tissues of male and female salmon during the spawning periods.

We have found the highest content of lipids and phosphatides in roe, followed by intestine, muscle, milt, liver, blood.

In roe, milt, liver, and blood the phosphatides make up 50-60% of the total quantity of lipids; in muscle and intestine it does not exceed 20% of the total quantity of lipids.

Choline is present in greatest amount in the roe.

The salmon does not differ from mammals in phosphatide content or in ratio between the amount of choline and total amount of phosphatides. The organs and tissues of males and females do not differ in content of lipids, phosphatides, and choline, except for the roe and milt.

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THE MECHANISM OF DETOXIFICATION OF TOXINS BY FORMALIN

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Translated from Biokhimiya Vol. 25, No. 1, pp. 28-33, January-February, 1960

Original article submitted April 8, 1959

The first information on the aggregation of nitrogenous products in toxoid formation was presented in 1925 by Karykin and co-workers [1] who observed that "fall in toxicity in treatment with formalin goes hand in hand with a decreased degree of dispersity." In 1926 Belozovich and Shneerson [2] found that when diphtheria toxin was detoxified by formalin there was a decrease not only in amino acid content, but in all the residual nitrogen. Such a decrease in amino nitrogen could depend on the accumulation of high molecular weight peptides.

In previous work [3] we have shown that when formalin acts on tetanus toxin there is aggregation of the nitrogenous products and accumulation of high molecular weight compounds, and the intensity of this process permits us to estimate the dynamics of the detoxification. In 1924, Loiseleur [4, 5] observed that when formalin acted on toxins there was a change in molecular structure of the nitrogenous products with an increase in fluorescence and a change of the spectral characteristics. The change in molecular structure of the nitrogen compounds under the action of formalin was nonspecific for the toxin because when formalin acts on amino acids, according to the data of Putnam [6], methylene cross linkages can be formed between amino groups on the one hand and amide, guanidine, phenol, and imidazole radicals on the other.

Our material contradicted the widely held view that the mechanism of toxoid formation consists in the methylene reaction of active amino groups of the toxin.

The purpose of the present work is to study the aggregation of the nitrogenous products in the preparation of diphtheria toxoid and to study in model experiments the aggregation of commercial peptones and amino acids in their reaction with formalin.

MATERIALS AND METHODS

Diphtheria toxin was obtained on Marten medium by growing diphtheria bacilli PW-8 for seven days under industrial conditions. Transformation of toxin into toxoid was carried out under industrial conditions at 37° over 21 days with 0.4% formalin.

In the model experiments we studied 1% and 5% aqueous solutions of commercial peptones (Eupeptone, Witt Peptone, Difco) and 10% aqueous solutions of amino acids: glycine, α- and β-alanine, valine, leucine, norleucine, cystine, cysteine, asparagine, β-phenylalanine, tyrosine, histidine, and lysine. The pH of the amino acid solutions was determined by the nature of the amino acids themselves except for tyrosine and cystine, which were dissolved in 5% sulfuric acid.

Treatment of the peptones was carried out for 15 days with 0.2, 0.4 and 2% solutions of formalin at 37°. In order to obtain the maximum aggregation of the amino acids, we used their concentrated solutions; treatment of the amino acids was carried out with a 2% formalin solution for 5 days at 37°. However, in a number of cases this temperature was found to be insufficient for aggregation and we then carried out a supplementary 2-hour boiling of the mixture of the amino acids with formalin on the water bath. In all the experiments we used formalin which contained 36.5% formaldehyde.

Fractionation of the nitrogenous products was carried out as described for the tetanus toxin [7]. The fractionation of the native diphtheria toxin and toxoid was carried out in the same way. The method of fractionation was as follows: the toxin protein was precipitated with 3% trichloroacetic acid fraction I. In the supernatant liquid the trichloroacetic acid concentration was brought to 10% which then precipitated fraction II, the high molecular weight fraction A. The supernatant liquid obtained after precipitation of fraction II was treated with 1% phosphomolybdic acid. This precipitated an intermediate molecular weight, biuret-giving peptide, fraction III. Fraction IV which did not precipitate with phosphomolybdic acid, consisted of amino acids, low molecular weight peptides, and heterocyclic compounds.

The fluorescence of the toxin and toxoid, peptides, and amino acids was determined photometrically with a specially adapted photometer [8]. The amino nitrogen of the diphtheria toxin and toxoid was determined by Sorensen formol titration.

RESULTS

Aggregation of Peptides when Diphtheria Toxin is Transformed to Toxoid

Samples for analysis were taken every three days. We studied 12 series of toxoids. The results of these determinations are given in Table 1.

When diphtheria toxin was incubated with formalin (Table 1) there was a regular increase in content of high molecular weight nitrogenous products at the expense of low molecular weight peptones and amino nitrogen. It

TABLE 1. Ratio of Nitrogenous Fractions of Diphtheria Toxin and Their Changes during Detoxification by Formalin (average data given in mg% nitrogen)

Length of toxin incubation with formalin	Fraction numbers				Amino nitrogen
	I	II	III	IV	
Before incubation	16,2	26,0	184	115,0	84
3 days	17,3	28,4	187	108,5	41
6 days	18,0	29,7	192	102,5	39
9 days	19,1	32,0	196	94,1	39
12 »	22,0	32,0	196	91,2	36
15 »	22,2	34,0	200	85,0	34
18 »	22,5	34,2	202	82,5	34
21 days	24,0	35,6	204	77,6	34

should be noted that we do not speak of free amino acids, but of amino groups, determined by Sorensen formol titration. The intensity of increase of the molecule of nitrogenous products during formation of diphtheria toxoid is considerably less than in the detoxification of tetanus toxin. This depends on the difference in concentration of peptones in the starting medium used for growing the diphtheria and tetanus bacilli: Marten peptone is less concentrated than Gluzman peptone.

Thus, in obtaining both tetanus and diphtheria toxoid there is a regular enlargement of the molecules of nitrogenous products as a result of direct reaction of the peptones and amino acids of the native toxin with formalin. We were interested in whether the combination of the peptones was specific for the toxin or whether it could occur by the addition of formalin to any peptone solution.

Aggregation of Nitrogenous Products in the Treatment of Amino Acids and Peptides with Formalin

When a 1% solution of peptone was greated with 0.2-0.4% formalin at 37° for 15 days the process of aggregation of the peptone occurred very intensively, and when a higher concentration of formalin (2% was used, combination of the peptides occurred especially rapidly. However, the intensity of the process in a solution of commercial peptones depended not only on the concentration of formalin, but also on the amount of peptone. The higher the concentration of the peptone solution, the more intensive was the accumulation of high molecular weight peptides. Therefore, when we treated a 5% solution of peptone with 2% formalin at 37°, even on the third day there was a sharp rise in the content of nitrogenous fractions I, II, and III, as well as even a precipitate of insoluble, abundant, flocculent and sticky material. When this precipitate had been air dried, it did not dissolve in water, alcohol or acids, dissolved slightly in 20% NaOH, and was a substance of a nonprotein character. The unusual chemical stability of this product and the substances obtained by formalin combination which remained in solution can explain the considerably greater stability of toxoid towards keeping than is shown by the original toxin.

Cheyroux [9] showed that the rate of detoxification of tetanus toxin by formalin depends on the pH of the medium and is a maximum in neutral and slightly alkaline media. It seems interesting to determine the relation of intensity of aggregation of the peptide to the concentration of hydrogen ion. It was shown that the maximum accumulation of high molecular weight products when a 1% peptone solution was treated with 0.4% formalin at 37° occurred at pH 7-8. At pH below 2 aggregation did not occur, and at pH above 9.5 it weakened. In the range of values from 4.5 to 8 the degree of aggregation changed slightly; at pH 4.5-7.0 it was lower than at pH 7-8. Thus, the intensity of aggregation depends on pH, temperature, concentration of formalin and of nitrogenous products.

If we assume that the aggregation of the peptides occurs because of combination of the terminal amino groups, we should try to explain which amino acids are able to form high molecular weight products in the presence of formalin. The aggregation of amino acids can be judged by the amount of precipitation of products by 1% phosphomolybdic acid in the presence of trichloroacetic acid, since phosphomolybdate under these conditions precipitates only high molecular weight compounds. Trichloroacetic acid does not precipitate aggregation products of amino acids.

As a result of experiments with amino acids, we found that glycine, α-alanine, valine, β-phenylalanine, and combinations of these amino acids in treatment with formalin do not give an aggregation reaction and are not precipitated by phosphomolybdic acid. Leucine, norleucine, cysteine, and asparagine after treatment with formalin precipitate to a very slight extent. The remaining amino acids studied, after incubation with formalin, are precipitated by phosphomolybdate.

The results of these experiments are given in Table 2.

The amino acids listed in the table in the presence of formalin can combine into more or less high molecular weight aggregates which are precipitated by phosphomolybdate. This aggregation occurs especially intensively, which is explained by the high concentrations of reacting amino acids and formalin, and by the high temperature of incubation. In practice when toxin is treated with formalin

TABLE 2. Precipitation of Amino Acids before and after Formalin Treatment (in mg% nitrogen of the precipitate)

Amino acids	Concentration of amino acids, %	Precipitate by addition of phosphomolybdic acid		
		before formalin treatment	after 5 day formalin treatment at 37°	after further 2 hour boiling
β-Alanine	10	—	87	262
Lysine	10	—	167	840
Cystine	10	—	41	41
Tyrosine	10	—	134	195
Histidine	4	—	109	920

the concentration of reacting reagents is less and the corresponding intensity of aggregation does not reach the same high value. Also, in the native toxin it is not chiefly the free amino acids which react, but the terminal amino acids of the peptides which in uniting give the high molecular weight products because of lengthening of the peptide chains. The model experiments with amino acids permit us to explain which of these products can undergo aggregation of the peptides.

The substances obtained in the formalin treatment, combined by methylene bridges, are not split by proteolytic enzymes of the tetanus bacilli and are not digested by 5% pepsin at pH 3.0 or by 5% pancreatin at pH 7.0. They do not coagulate when heated and are intensely fluorescent. The aggregation products of amino acids do not give a biuret reaction; the biuret reaction of peptides treated with formalin is not changed. Just like the spectral characteristics. These results indicate that the resulting substances have a nonprotein character and are polymers of the original substances.

DISCUSSION

We can conclude from the foregoing that in toxoid formation the process of increase in the molecule size is nonspecific and can occur not only with toxins, but with any peptone. The aggregation of the peptones occurs apparently by reaction of the terminal amino acids with formalin as a result of which strong bonds are formed. The combination of amino acids into high molecular weight compounds has been shown for β-alanine, lysine, cystine, tyrosine, and histidine. The products resulting from such a combination are characterized by stability and chemical inertness. They fluoresce intensely which explains the fact observed by us [8] of the increase in fluorescence in toxoid formation. These results, and also those described earlier [3] on the parallelism between detoxification of the toxin and accumulation of high molecular weight products under the ordinary conditions of toxoid formation, and also the change with concentration of formalin, temperature, and pH permit us to draw the following conclusions.

The basic mechanism of detoxification of toxins by formalin is the aggregation of nitrogenous products, at which time there occurs binding of the toxophoric groups of the toxin. It is possible that there is a combination of formalin with separate groups of the toxin without formation of methylene bridges, since some amino acids do not combine with formalin, but this mechanism is less probable.

Diphtheria and tetanus toxins, like all proteins, are constructed of peptide bonds. Free amino groups in such proteins can be provided by N-terminal amino acids and also by free amino groups of diamino acids which have not formed acid amide bonds. Such free amino groups are obviously many times more frequent in each protein molecule than terminal amino groups, and it is just these diamino acids and cyclic amino acids that are particularly easily aggregated by formalin. This is why we can assume that the basic mechanism of toxoid formation is a combination of molecules of nitrogenous products.

Such as assumption allows us to explain the data on dynamics of toxoid formation.

Thus, Bobkova [10] found that most of the formalin combined with diphtheria toxin rapidly, while Belozovich and Shneerson [2] noted that detoxification of diphtheria toxin occurred gradually, and at the end of the first day of toxoid formation the preparation contained a considerable quantity of undetoxified toxin. It is known that the addition of formalin to amino groups in the Sorensen type of reaction occurs in the cold and toxoid formation occurs in the thermostat, that is, under conditions in which formalin not only combines with amino groups, but also leads to aggregation of the molecules of nitrogenous substances. These findings contradict the theory of detoxification of toxin by adding formalin to the amino group.

The data obtained in the model experiments, of incubating peptones with formalin permit us to explain a number of long known facts. Thus, the stability of diphtheria and tetanus toxoids to long keeping [11, 12] and to relatively high temperature (heating 70° for 1 hour and 80° for 20 min [12]) are easily explained by the chemical inertness of the polymeric structure of the toxoid.

All these facts indicate the impossibility of reconsidering the theory of toxoid formation, and favor the assumption

of the aggregation of molecules of nitrogenous substances in the formalin treatment.

SUMMARY

During toxoid formation there occurs an increase in size of the molecules of nitrogenous products. This increase in size is explained by the aggregation of amino acids and peptides through methylene bridges. The process of increase in size of the molecules occurs not only toxins, but with any peptones.

The combination of amino acids into high molecular weight compounds has been shown for β-alanine, lysine, cystine, tyrosine, and histidine. These amino acids, after incubation with formalin, acquire the ability to fluoresce intensely and are precipitated by phosphomolybdic acid in the presence of trichloroacetic acid.

There is a definite parallel between the intensity of detoxification of the toxin and the accumulation of high molecular weight products. It is possible that the combination of the molecules of nitrogenous products is the basic mechanism of detoxification of toxins. The substances obtained as a result of aggregation through methylene bridges are chemically inert, stable, and polymeric. One of the satisfactory methods of finding the aggregation of nitrogenous products is fractionation with trichloroacetic acid and phosphomolybdic acid.

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THE PROBLEM OF INTERMEDIATE NUCLEOTIDE METABOLISM IN NORMAL AND X-RAY IRRADIATED BONE MARROW

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Translated from Biokhimiya Vol. 25, No. 1, pp. 34-42, January-February, 1960

Original article submitted April 20, 1959

The study of intermediate nucleotide metabolism in bone marrow is of special interest, because:

1) Bone marrow is suitable for study of the general rules of intermediate nucleic acid metabolism, since the biosynthesis of these acids occurs very rapidly in bone marrow as compared to the rate and biosynthesis in most other animal organs.

2) Nucleic acid metabolism in bone marrow is very sharply damaged by various injuries to blood production, and especially by the action ionizing radiation [1, 2]. Preliminary data on intermediate nucleotide metabolism in bone marrow in normal marrow and after x-ray irradiation has been published previously [3-5]. In the present communication we give data on identification of free nucleotides of bone marrow and the effect of x-ray irradiation on their content, and also evidence for the presence of the reaction of orthophosphorolysis of ribonucleic acids during the autolysis of bone marrow in an orthophosphate buffer solution.

METHODS

In the experiments with x-ray irradiation, to remove errors from individual variations in the comparison of irradiated and nonirradiated bone marrow, we irradiated one (right) rear leg of a rabbit. The rest of the body was screened with 55 mm thick lead. The score of x-ray irradiation was an RUP tube. Conditions of irradiation: 180 kv, 15 ma; λ-15 A, filters 0.5 mm Cu and 0.75 mm Al. Duration of irration 21 min, dose 200 r. Two days after the irradiation, when the damage to the irradiated bone marrow was clearly shown, the rabbit was sacrificed by decapitation, exsanguinated, and the bone marrow was rapidly removed in a cold room (3°).

In the bone marrow homogenate we determined the content of preformed nucleotides and also the enzyme activity. For a determination of phosphates by paper chromatography, the chromatograms were sprayed with a mixture of ammonium molybdate and ascorbic acid. These conditions were selected on the basis of the Lowry method [6] for the determination of phosphorus in solution. The solution for spraying the chromatogram was prepared by mixing a 0.15% ammonium molybdate solution in 0.6 N H_2SO_4 and a 10% ascorbic acid solution. The solutions were mixed in the relative proportions of 17 : 1 immediately before spraying the chromatogram. After the chromatogram had been sprayed, there rapidly developed on it a grayish blue spot of orthophosphate, and about an hour after spraying there appeared violet spots of pyrophosphate and metaphosphate. After still longer keeping of the chromatogram, blue-violet spots of adenosine triphosphate, adenosine diphosphate, and adenylic acid also became visible. The limits of sensitivity of this method were 0.05 γ P for orthophosphate and 0.5 γ P for pyrophosphate.

For determination of the content of preformed nucleotides, and also for determination of the products of breakdown of nucleic acids in the bone marrow homogenate, we used two main methods for preparing the extracts to be analyzed: extraction of the nucleotides with 60% ethanol heated to boiling [4], and extraction with 0.6 N perchloric acid [7]. The extracts were analyzed spectrophotometrically in the ultraviolet, thus determining deoxyriboses according to Barton [8] and riboses according to Albaum and Umbreit [9].

In some experiments we modified the Barton method as as follows: The solutions to be analyzed were first brominated according to Massart and Hoste [10]. This permitted determination by the Barton method not only of purine, but also of pyrimidine deoxyribose compounds.

The extracts were also analyzed by a paper chromatography and study of the fractions visible in the ultrachemiscope [11]. Comparison of the composition of the low molecular weight purine and pyrimidine compounds in the aqueous alcohol and the perchloric acid extracts according to the results of viewing the chromatograms in the ultrachemiscope showed the great similarity in the components of these extracts. Chromatography was carried out by the descending method using Leningrad "slow" paper which had first been washed with 25% formic acid. Length of chromatogram 45 cm. We used two mixtures as solvents. Solvent No. 1: a mixture of 95% ethanol-anhydrous butano-1 M ammonium acetate buffer with pH 3.7-3.8 in the ratio 4 : 1 : 2 [12]. Solvent No. 2: n-butanol saturated with water-85% formic acid in the ratio 9 : 1.

If the starting spot on the chromatogram was a line 9-10 cm long, it was possible on the chromatogram to "fractionate" a quantity of extract which corresponded to 1-2 g of bone marrow. The passage of solvent was continued to the lower edge of the chromatogram, then the chromatogram was removed from the chamber, dried, and

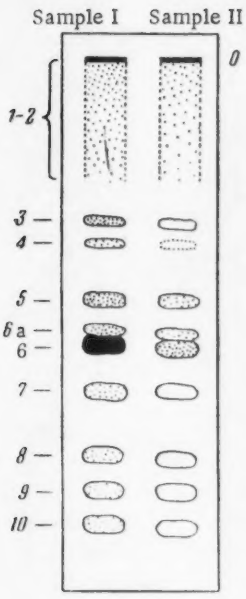

Fig. 1. Typical distribution pattern on the chromatogram of low molecular weight purine and pyrimidine compounds contained in bone marrow. From data of viewing the chromatogram in the ultrachemiscope. Solvent No. 1 was passed twice through the chromatogram. Figures to the left are the fraction numbers: 1-2) Unidentified nucleotide derivatives; 3) adenosine triphosphate (?); 4) adenosine diphosphate (?); 5) guanylic acid; 6a) inosinic acid; 6) adenylic acid; 7) uridylic acid; 8) unidentified fraction; 9) hypoxanthine; 10) uracil. Sample I) Normal; Sample II) two days after local irradiation with a dose of 2000 r (duration of irradiation 21 min). O) starting point of chromatogram.

again placed in the chamber and the same solvent was passed a second time to the lower edge of the chromatogram.

Preformed Free Purine and Pyrimidine Compounds

The results of the identification of the spots on the chromatogram from the perchloric acid extract of bone marrow are given in Fig. 1.

We give some concrete material on the use of the method of identification of these fractions. Fraction 5 in the series corresponds to guanylic acid. Typical results of chromatography of fraction 5 and its base are given in Fig. 2. The eluate of fraction 5 from the paper has an absorption maximum at 254-255 mμ (the theoretical absorption maximum of guanylic acid is 253 mμ). Fraction 5 gives a positive reaction for ribose with orcinol. Fraction 6a in the series corresponds to inosinic acid. Chromatography of the hydrolyzate of fraction 6a showed that the nucleic base of fraction 6a is hypoxanthine (Fig. 3). The ultraviolet absorption spectrum of fraction 6a shows a maximum which corresponds to the absorption spectrum of inosinic acid (250 mμ).

As we have already mentioned [4], parallel chromatography of fraction 6 with comparison with different ribonucleotides showed that this fraction corresponds to muscle adenylic acid. In the present investigation we have carried out chromatography of the nucleic bases obtained by the hydrolysis of fraction 6.

Hydrolysis of fraction 6 was carried out in 1 N HCl at 100° for 15 min. The hydrolyzate was evaporated on the water bath. Water was added to the dry residue and

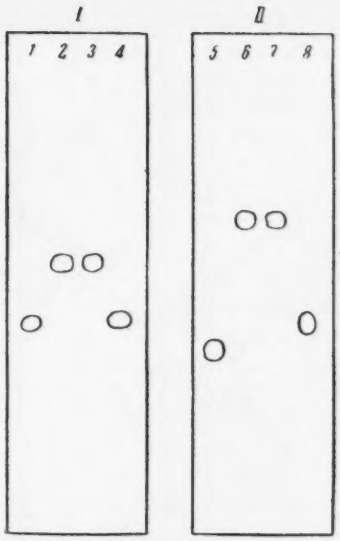

Fig. 2. Identification of fraction 5. Spots visible on the chromatogram in the ultrachemiscope after two passages of solvent. I) Chromatogram in solvent 2, fraction 5 after hydrolysis (1 N HCl, 15 min, 100°). Substance chromatographed: 1) Uridylic acid; 2) guanylic acid; 3) fraction 5; 4) cytidylic acid; 5) hypoxanthine HCl; 6) guanine HCl; 7) fraction 5 after hydrolysis in 1 N HCl; 8) adenine HCl. According to the data of paper chromatography, fraction 5 corresponds to guanylic acid.

it was again evaporated dry. The dry residue was dissolved in a small amount of water and placed on the chromatogram on which had also been deposited a series of standards in the form of adenine chloride, guanine, and hypoxanthine.

Chromatography both in solvent No. 1 and solvent No. 2 showed the agreement of the nucleic base of fraction 6 with adenine (Fig. 4), which confirmed their identity. The ultraviolet absorption spectrum of fraction 6 particularly agreed with the absorption spectrum of muscle adenylic acid. The absorption maximum was found at 260 mμ.

The solution of fraction 6 gave a positive reaction for ribose with orcinol. In one experiment we eluted one of the two spots on the paper chromatogram of fraction 6 with water and found adenylic acid in the eluate; according to the data of ultraviolet spectrophotometry, 0.9 mg; according to the results of the orcinol reaction, 1.1 mg. It is possible that in fraction 6, besides adenylic acid there is a considerably smaller amount of other substances.

The results of chromatography of fraction 7 are given in Fig. 5. As can be seen, fraction 7 under the conditions of separation migrates with uridylic and cytidylic acids and differs sharply in mobility from guanylic acid.

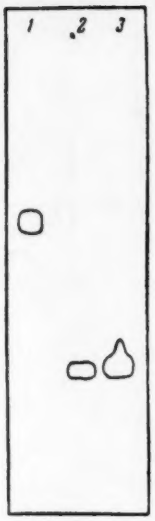

Fig. 3. Identification of the purine base of fraction 6a. Chromatography in solvent 2 of the acid hydrolyzate of fraction 6a. Spots seen on the chromatogram in the ultrachemiscope: 1) Hydrolyzate of guanylic acid in HCl; 2) hydrolyzate of fraction 6a in HCl; 3) hypoxanthine HCl. The solvent was passed twice through the chromatogram.

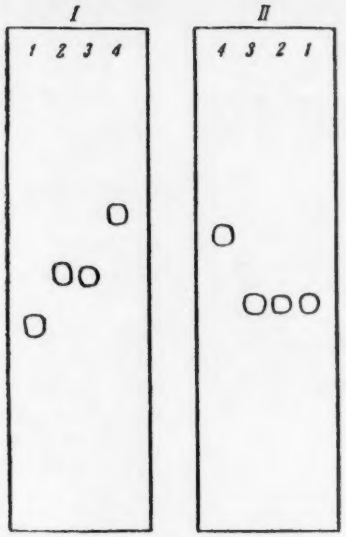

Fig. 4. Identification of nucleic base of fraction 6. Spots visible in the chromatogram in the ultrachemiscope after chromatography of the acid hydrolyzate of fraction 6 in parallel with the standard. Chromatograms I and III) after twice passing solvent No. 2. Chromatogram II) after one passing of solvent No. 1. 1) Hypoxanthine; 2) nucleic base of fraction 6; 3) adenine; 4) guanine; 5) uracil.

The position of fraction 7 below fraction 6 and the correspondence of fraction 6 with adenylic acid indicates that fraction 7 is not adenylic acid. Cytidylic and uridylic acids differ from each other considerably in the position of their absorption maxima. Uridylic acid has an absorption maximum at 262 mμ and cytidylic at 271 mμ, in a neutral medium. The aqueous eluate of fraction 7 has an absorption maximum at 261 mμ, which practically agrees with the absorption maximum of uridylic acid and differs markedly from the absorption maximum for cytidylic acid. From the position of the absorption minimum of fraction 7 it also corresponds to uridylic acid (232 mμ) and does not correspond to cytidylic acid (250 mμ). After 15 minutes hydrolysis in 1 N HCl at 100° the nucleic base of fraction 7 was split slightly, which is characteristic for pyrimidine and not for purine nucleotides. Thus, fraction 7 corresponds to uridylic acid.

Chromatography of fractions 9 and 10 with standards showed that fraction 9 is hypoxanthine and fraction 10 is uracil (Fig. 6).

The eluate from fraction 9 has an ultraviolet absorption maximum of 249 mμ (theoretical maximum for hypoxanthine 250 mμ). The eluate from fraction 10 has an ultraviolet absorption maximum of 260 mμ (theoretical for uracil, 260 mμ). This close agreement also shows that fraction 9 is hypoxanthine and fraction 10 is uracil.

Fractions 3 and 4 were not finally identified. Fraction 3 in position on the chromatogram and in spectrum corresponds to adenosine triphosphate and fraction 4 on the same evidence corresponds to adenosine diphosphate. In acid hydrolysis of fractions 3 and 4 a base is formed identified as adenine on the chromatogram. However, it is possible that in these fractions there are contaminants and other substances. Especially in fraction 3 there are possible admixtures of diphosphopyridine nucleotide which would occupy the position on the chromatogram which corresponds to fraction 3.

Fraction 8 remains unidentified, its ultraviolet absorption is 250 mμ. When two different solvents are used on the chromatogram, it is shown that this fraction contains at least two substances.

Deoxyribose compounds, by the Barton reaction, are present only in traces in the bone marrow extracts.

Thus, in one experiment 5 g of bone marrow can be extracted in the cold with double the volume of 0.6 N $HClO_4$ with subsequent precipitation of the latter from the filtrate by adding a cold concentrated KOH solution to the point of neutrality. In 1.5 ml of filtrate from the $KClO_4$ precipitate we found an amount of deoxyribose corresponding to 3.4γ DNA. In another experiment this value was zero.

Thus, even concentrated acid extracts from bone marrow contain practically no purine deoxyribonucleotides. This was also confirmed by determination of deoxyribose according to Barton after preliminary bromination of the extracts. Thus, in 3.5 g of bone marrow the amount of deoxyribose compounds was zero, with a sensitivity of the method of 3-4 μ g of deoxyribose compounds.

Bromination of the pyrimidine deoxyribose compounds also makes possible their satisfactory determination with diphenyl amine, like the purine deoxyribose compounds [13].

In Table 1 we give the results of the study of the action of x-ray irradiation on the content of acid soluble purine and pyrimidine compounds in bone marrow.

Thus, in the period of severe damage to the blood caused by irradiation of bone marrow, the content of, the

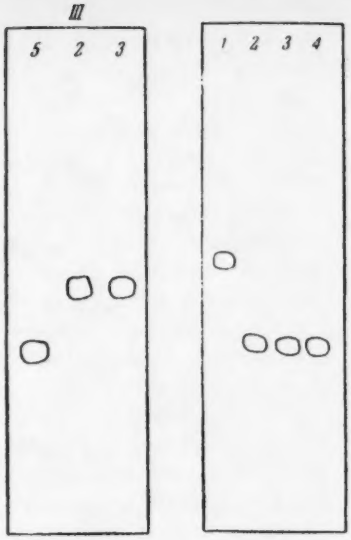

Fig. 5. Identification of fraction 7. Spots visible on the chromatogram in the ultrachemiscope after twice passing solvent No. 1. Substances chromatographed: 1) Guanylic acid; 2) fraction 7; 3) uridylic acid; 4) cytidylic acid. (Explanation in text).

content of low molecular weight purine and pyrimidine compounds is markedly lessened.

Changes in Content of Free Purine and Pyrimidine Compounds in Bone Marrow during Autolysis

Study of the changes in content of free purine and pyrimidine compounds in bone marrow during autolysis is of considerable interest from the point of view of explaining the method of splitting of nucleic acids. As is known, the reaction of phosphorolysis of RNA was discovered comparatively recently in bacteria, and the possibility was also established of the synthesis of DNA by the reaction of "retropyrophosphorolysis," that is, the reverse reaction of pyrophosphorolysis. In this connection it is of interest to study the autolytic accumulation of nucleotide compounds in bone marrow in ortho- and pyrophosphate buffer solutions.

The study of the processes of splitting of nucleic acids under the influence of autolysis allows us to investigate the splitting under conditions in which the structure of the nucleic acids corresponds to the specificity of the enzyme systems which govern their biosynthesis.

Perhaps the role of these facts is considerable. Thus, our investigations showed that DNA from herring sperm is split by extracts of bone marrow in a pyrophosphate buffer solution considerably more intensely than in an orthophosphate buffer solution. Along with this, splitting of thymus DNA on the contrary is considerably more intense in an orthophosphate than in a pyrophosphate buffer solution. The action of x-ray irradiation on autolytic splitting of nucleic acids has not yet been studied, as far as we know, although it is evident that changes in this process under the action of x-ray irradiation may be of great significance in radiation sickness.

Autolysis of the bone marrow homogenate was carried out in 0.3 M buffer solutions at 38° for one hour. We ran parallel controls without incubation. Typical conditions

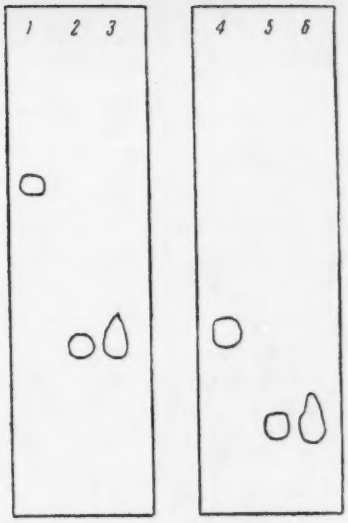

Fig. 6. Identification of fractions 9 and 10. Spots visible on the chromatogram in the ultrachemiscope after twice passing solvent No. 2. 1) Guanine; 2) fraction 9; 3) hypoxanthine; 4) adenine; 5) fraction 10; 6) uracil. Further explanation in text.

of autolysis of bone marrow homogenate are given in Table 2.

After incubation (in the control, without incubation) the proteins and nucleic acids were precipitated by addition in the cold of 1.2 N $HClO_4$, 3.5 ml per sample. After 30 min, the precipitate was filtered, and the filtrate was neutralized with concentrated KOH. The precipitate of $KClO_4$ was filtered off, and the filtrate was analyzed by ultraviolet absorption, by ribose content by the method of Albaum and Umbreit [9], and by deoxyribose content according to Barton [8]. Deoxyribose compounds were scarcely found in either the orthophosphate or the pyrophosphate buffer solutions: the extinction values were at the border of sensitivity of the method of study.

According to the results of the determination of ultraviolet absorption and of ribose by the orcinol reaction, the autolytic splitting of ribonucleic acid performed in the bone marrow and was several times higher in the orthophosphate buffer than in the pyrophosphate one.

After x-ray irradiation the autolytic splitting of the ribonucleic acid was suppressed 2-3 fold in both orthophosphate and pyrophosphate buffers (Fig. 7).

These facts indicate that in normal and irradiated bone marrow there is probably a reaction of phosphorolysis of RNA along with its hydrolytic splitting.

Since no measurable accumulation of low molecular weight deoxyribose compounds was found in autolysis, then in all probability under these conditions there is splitting of RNA but not of DNA. Orienting calculations showed that after one hour incubation in an orthophosphate buffer about 15% RNA of normal bone marrow is split. The data

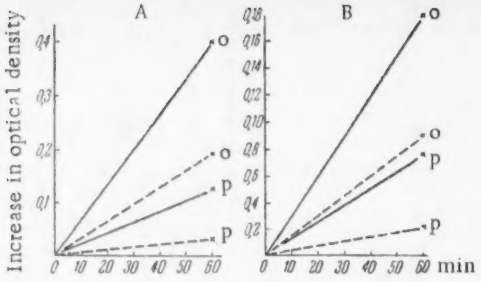

Fig. 7. Autolytic decomposition of nucleic acids in bone marrow; normal sample and after x-ray irradiation. A) From ultraviolet absorption data. Average of 4 experiments. B) From ribose determination. Average of 3 experiments. O) Autolysis in orthophosphate, P) in pyrophosphate. Solid line, normal; dotted line, after irradiation.

shown, the aqueous alcoholic extract is not suitable for determination of inorganic pyrophosphate in bone marrow, since 60% alcohol precipitates inorganic pyrophosphate. In chromatography of the perchloric acid extract of bone marrow and treatment of the chromatogram with a mixture of molybdate and ascorbic acid by the method described above inorganic pyrophosphate is not detected. Control experiments showed that inorganic pyrophosphate added to the bone marrow homogenate with subsequent one hour incubation was not markedly split. The almost complete absence of inorganic pyrophosphate in bone marrow indicates that the reaction of pyrophorolysis or retropyrophosphorolysis under natural conditions is practically absent.

There is information in the literature on the inhibiting action of irradiation on the uptake of different precursors into nucleic acids. However, these results have not yet shown the presence of a sharp suppression of enzyme systems which assure the polymerization of the immediate

TABLE 1. Content of Purine and Pyrimidine Compounds (Fractions) in Perchloric Acid Extracts of Rabbit Bone Marrow, Normal and Two Days after Irradiation. Irradiation dose 2000 r. Content of fractions expressed as extinction at maximum point per 1 g wet weight of bone marrow. Volume of eluate, 5 ml

Experimental conditions	Fraction No.									
	1--2	3	4	5	6a	6	7	8	9	10
Normal	0,142	0,050	0,054	0,150	0,250	0,719	0,083	0,019	0,092	0,090
Irradiated	0,123	0,023	0,000	0,075	0,083	0,424	0,039	0,018	0,022	0,024
Irradiated, in % of normal	87,7	46	0	50	33	59	47	95	24	27

of determination of autolysis by ultraviolet absorption are apparently closer to the true values of nucleic acid splitting than are the data from the determination of ribose.

The combination of the resulting data still does not give a basis for assuming that in bone marrow there is a reaction of retro pyrophosphorolysis of DNA [14]. The question of the immediate precursors of DNA in bone marrow has not yet been sufficiently worked out. It is possible that deoxyribonucleotides and pyrophosphate are present in bone marrow in extremely small amounts which are difficult to detect by the present methods.

The almost complete absence of low molecular weight deoxyribose compounds in bone marrow indicates the possibility of synthesis of DNA from ribonucleotides with their reduction into deoxyribonucleotides in an already combined state. The results of Potter and coworkers [15] are of some interest in connection with acid soluble thymus nucleotides. He was unable to find in the thymus any free purine deoxyribonucleotides, although in the thyroid, as in bone marrow, DNA synthesis is very intense.

For an explanation of the possibility of the reverse reaction of pyrophosphorolysis of nucleic acids in bone marrow there is interest in the question of the presence of inorganic pyrophosphate in bone marrow. As we have

precursors of the nucleic acids. The inhibition may depend on an insufficient quantity of other substances which are taken up into the nucleic acids at the same time. Since we can assume that during autolysis the splitting of RNA of bone marrow in an orthophosphate buffer solution occurs by the reverse process of orthophosphorolysis of RNA, the fact of reduction in autolysis by radiation under these conditions evidently shows the inhibition after radiation of the activity of the enzyme systems which synthesize RNA from its direct precursors. However, we should remember that the inhibition of autolytic splitting of nucleic acids after irradiation could also depend on qualitative or quantitative changes in the substates themselves, the nucleic acids.

Thus, it is known that molecules of nucleic acid under the influence of irradiation can either be depolymerized [16] or give a "stitching" together of the separate chains [17]. Also, after irradiation there may occur changes in the composition of the nucleic acids themselves [18].

SUMMARY

In rabbit bone marrow we have found: adenylic, inosinic, guanylic, and uridylic acids, hypoxanthine, uracil, and other low molecular weight purine and pyrimidine substances. The content of these substances is

TABLE 2. Conditions of the Experiments on Autolytic Splitting of Nucleic Acids of Bone Marrow in Normal Subjects and after Irradiation

Components	Normal				Two days after irradiation with 2000 r			
	with orthophosphate		with pyrophosphate		with orthophosphate		with pyrophosphate	
Bone marrow homogenate, g	0,3	0,3	0,3	0,3	0,3	0,3	0,3	0,3
0.3 M orthophosphate buffer solution, pH 7.0 ml	3,5	3,5	0	0	3,5	3,5	0	0
0.3 M pyrophosphate buffer solution, pH 7.0 ml	0	0	3,5	3,5	0	0	3,5	3,5
Incubation, min	0	60	0	60	0	60	0	60

sharply decreased in two days after local x-ray irradiation at a dose of 2000 r. We have also found traces of low molecular weight preformed deoxyribose compounds. We have observed that pyrophosphate is completely absent. This shows that the reaction of pyrophosphorolysis is practically absent in bone marrow under natural conditions.

The autolytic splitting of RNA performed in bone marrow is 2-3 times faster in orthophosphate buffer solution than in pyrophosphate. This shows the possible presence of a reaction of orthophosphorolysis of RNA along with hydrolytic splitting of RNA. The enzymatic splitting of RNA performed in bone marrow in ortho and pyrophosphate buffers two days after local irradiation at a dose of 2000 r is decreased 2-3 fold.

The Barton method for determination of deoxyribose compounds has been modified as follows: the solution to be analyzed is first brominated by the method of Massart and Hoste. This permits determination by the Barton method not only of purine deoxyribose compounds, but also of pyrimidine deoxyribose compounds.

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THE EFFECT OF THE CONFIGURATION OF THE DEOXYRIBONUCLEIC ACID MOLECULE ON THE BIOLOGICAL ACTIVITY OF THE PROTEIN BOUND TO IT

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Translated from Biokhimiya Vol. 25, No. 1, pp. 43-54, January-February, 1960

Original article submitted April 22, 1959

We have showed in previous studies [1] that when high molecular weight preparations of deoxyribonucleic acid (DNA) react with proteins there is a definite relation between the amounts of the components which combine and the size of their molecular weights, that is, the amount of protein which combines with DNA (expressed in moles) is directly proportional to the molecular weight of DNA and inversely proportional to the molecular weight of protein.

A study of the changes in characteristic viscosity and relative relaxation of the DNA-protein compound with gradual increase in amount of protein in it shows that the protein is combined with the DNA apparently in the form of an unusual monolayer in which each protein molecule has a point of contact with DNA [2]. However, the experimentally observed maximum amount of protein [1] combined with DNA (100 moles of beef serum albumin and 2900 moles of α-chymotrypsin per mole of DNA with molecular weight $3.5 \cdot 10^6$), as was shown by calculation of the ratio of surfaces of DNA and protein molecules [3], could not be spread as a monolayer on the DNA molecule either in the native form or as a fully extended (β-configuration) molecule. The distribution of the protein molecule on the axis perpendicular to the axis of the bispiral DNA structure was excluded by measuring the change in asymmetry of the molecule. On the basis of our data we suggested that part of the protein was combined with the DNA in the form of a fully extended β-configuration molecule (the amount of this protein was calculated to be 15-25%) and another part of the protein was apparently distributed on the DNA molecule in the α-configuration with some sort of deformation on the DNA surface and with poorer packing than on the native molecule. (The idea of the presence of protein in the form of the extended β-configuration in nature and the "reconstituted" nucleoprotein was indicated by the data of x-ray analysis [4].)

It can be assumed that differences in type of spacial distribution of the protein molecules in an artificially obtained nucleoprotein should also lead to different biological activities in various configurations of the proteins. The study of this question is the subject of the present paper. The effect of DNA on the activity of the proteins bound with it was studied in two model systems in the artificially obtained compounds of high molecular weight DNA with α-chymotrypsin and with insulin.

METHODS

The preparation of high molecular weight DNA was obtained from freshly frozen pancreas and liver of cattle and from calf thyroids by the modified method of Mirsky and Pollister [1, 5]; deproteinization was carried out by the method of Sevag [6].

The entire operation was carried out at 2-4°. The concentration of the preparation in the DNA solution was determined by the phosphorus content and the degree of purification from protein by the N/P ratio; phosphorus was determined according to Fiske-Subbarow, nitrogen according to Conway after mineralization with H_2SO_4. The molecular weight of DNA was determined viscosimetrically [7]. The characteristic viscosity was measured in a 0.2 M NaCl solution under equalized conditions of DNA concentration in an Ostwald viscosimeter with a capillary diameter of 0.6 mm, length 10 cm, at 25 ±0.01°. The resulting DNA preparation had the following properties: N/P = 1.79; 1.70, 1.69. Molecular weight $3.5 \cdot 10^6$; $3.41 \cdot 10^6$; $2.0 \cdot 10^6$.

Crystalline α-chymotrypsin was obtained by the method of Kunitz and Northrop. The preparation after dialysis was submitted to lyophilic drying. The compounds of DNA with α-chymotrypsin (in the form of the monomer) and with insulin were obtained by the method described earlier [1]; in the experiments we used a commercial insulin preparation, 2 mg/ml, 40 units. Obtaining a given protein content in the complex was assured by varying the conditions of the relative concentrations of DNA and protein in the reaction medium from 1 : 0.2 to 1 : 600 [1, 3]. The compounds which were formed were characterized by their protein content (based on N), their nucleic acid (based on phosphorus), and by the characteristics viscosity of the solutions, determined, as mentioned, in 0.5 M NaCl solution.

The proteolytic activity of the α-chymotrypsin was determined by the method of Anson, with incubation of different amounts (calculated on chymotrypsin) of free enzyme and enzyme bound to DNA in a solution of 1/15 M phosphate buffer pH 8.04, at 37°, under the same conditions of enzyme and substrate concentration in the medium, also equalized by NaCl concentration in the experiment and the control (free chymotrypsin). The activity was determined by the amount of tyrosine liberated during

incubation with the substrate (colorimetric analysis of the trichloroacetic acid filtrate with Folin reagent in an alkaline medium on an FEK-M apparatus compared to control samples which contained the same components but without incubation).

The insulin activity was characterized by the hypoglycemic action, the relative decrease in amount of blood sugar of healthy rabbits after intramuscular injection of equal amounts of it (0.5 units/kg live weight of the rabbits) free or bound with DNA. Blood sugar was determined by the method of Hagedorn-Jensen.

EXPERIMENTAL

Proteolytic Activity of α-Chymotrypsin in the Complex with DNA

A study of the specific proteolytic activity of α-chymotrypsin bound in different ratios with DNA showed (Fig. 1) that the activity of the enzyme definitely depended on the amount of the protein contained in the complex. The enzyme showed 15 to 20% of the original activity (with respect to the rate of proteolysis of the free enzyme taken as 100%) in complexes which contained 40-45% protein; when the amount of the latter was raised the specific activity of the enzyme at first increased sharply, almost reaching the level of the control at 65-75%, but then falling markedly again, dropping to 40% at 90% protein content. *

The observed effect agrees well with our idea of the presence of a nonhomogeneous type of structural change in the protein fixed on the DNA molecule; thus, evidently, different configurations of the protein determine the different catalytic activities of the ratios of the different amounts of proteins extended on the DNA molecule determine the total activity of the enzyme in the complex.

In compounds which contain only 40-50% protein, most of it apparently occurs in the state of a fully extended molecule (β-configuration), since this type of compound, from steric considerations (distance between nucleotides and side groups of the extended polypeptide chain 3.4 A [8]) should predominate [3]. In such a distribution of protein – fully extended on a DNA molecule – all the basic groups of the protein will be bound with the phosphorus groups of DNA, and one of the active centers of the chymotrypsin, which according to some investigators is the imidazole ring of histidine [9], will thus be completely blocked. If extension of the form of the protein is connected with loss of enzyme activity (due to blocking or destruction to the topographic distribution of the reactive centers of the molecule and perhaps also to steric difficulties), then it is natural that complexes in which this type of packing predominates will have low proteolytic activity. Indirect evidence for this idea lies in the fact that extrapolation in the curve to zero activity (Fig. 1) gives a protein content on the order of 26-28%, that is, an amount which, by calculation, can be dispersed on a DNA molecule in the form of the extended β-configuration [3].

When the content of the protein in the molecule is raised, the relative amount of protein bound in the β-configuration is decreased; another type of spacial distribution of the protein dominates (evidently there is partial deformation of the protein molecule in the α-configuration) and the activity of the enzyme is equal to or higher than the activity of the free protein, which is reflected in the general increase in enzyme activity.

For an explanation of the right hand part of the curve (Fig. 1) which shows a further decrease in specific activity of the enzyme with rise in content of protein in the complex these two ideas on different configurations of the protein on the DNA molecule are obviously insufficient; here, in all probability, we must also consider the possibility of blocking the reactive centers of the molecule because of increased effect of intermolecular reactions, the effect of which rises especially with rise in content of protein in the complex above 75% [10, 3].

Biological Activity of Insulin Bound with DNA

The DNA-insulin complex was obtained in a citrate buffer, pH 4.2, with ionic strength 0.15. For the experiment we used the high molecular weight DNA (molecular weight $3.5 \cdot 10^6$) and the commercial preparation of insulin (2 mg/ml, 40 units). As the results of the experiments showed, insulin combines with DNA in different proportions. The amount of bound protein increases with increased starting ratio of protein and DNA in the reaction mixture. The resulting compound resembles in its properties the natural nucleoproteins; it has the ability to form relaxing fibers in physiological saline solution and shows the lowered viscosity of solutions characteristic for nucleoproteins. The characteristic viscosity of DNA in the compound with insulin just as in the compound of high molecular weight DNA with other proteins is twice as low as the characteristic viscosity of the initial DNA [1, 3]. As the results of the experiments showed, insulin combined with DNA retains its biological activity.† The dependence of the specific activity of the hormone on the amount of it in the complex with DNA is shown in Fig. 2. As these data show, the insulin activity shows a definite, regular dependence on the quantity of protein in the complex. At an insulin content of 44-50% the activity of the hormone compared to the activity of the free insulin has fallen to 26-30%; as the protein content in the compound rises, the activity increases and reaches the control level at 70-77%. Further increase in protein content in the complex results in a fall in the hypoglycemic effect of the hormone (to a

* It was shown in separate experiments that the action of DNA was not specific; high molecular weight preparations of DNA obtained from different sources (pancreas, liver, thyroid) had the same effect on the character of change in rate of proteolysis of the enzyme bound to them.

† The activity of insulin was determined in the Division of Pharmacology of our Institute by Chief Scientific Assistant E. A. Trutneva, to whom we express our thanks.

level of 60% when the protein content in the complex is 90%). Thus, when we study the biological properties of the DNA compound with insulin in experiments in vivo we find the same regularities as are found in the study of the DNA-α-chymotrypsin complex in the in vitro experiments.

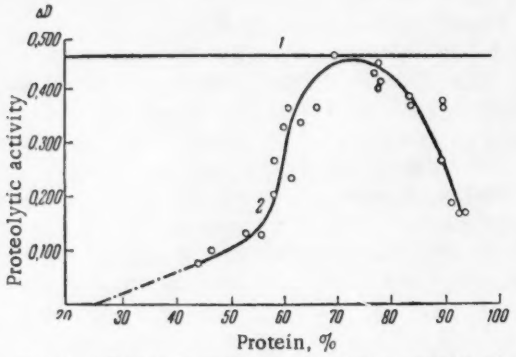

Fig. 1. Change in specific proteolytic activity of α-chymotrypsin bound to DNA depending on its content in the complex. 1) Specific activity of free chymotrypsin; 2) of chymotrypsin in the complex. Amount of enzyme in the experiment 0.25 mg, incubation 1 hour.

It is an interesting fact that the complexes of high molecular weight DNA with insulin are evidently stable enough, and specifically, the structure of the compound is not destroyed in the organism during a longer or shorter time (about 5 hours).

(In the case of dissociation of the bond in DNA-insulin, and also of depolymerization of DNA, the activity of the bound insulin would obviously equal that of the control). The circumstance is also worth noting that the insulin bound to DNA enters into metabolic reactions in the organisms as easily (at definite compositions of the complex) as free insulin. Figure 3 shows the curves of activity of free and bound insulin with time. These data show that insulin bound with DNA does not have a prolonged action, but on the contrary, insulin which has formed the complex loses its activity faster than does free insulin. However, in general the fall in insulin activity retains the regular dependence of the activity of the complex on the amount of protein in it. Evidently in this case the difference in hormonal activity of the protein bound in different ratios with DNA is well explained from the point of view of the presence of inhomogeneities in the structural organization of protein on the DNA molecule which determine the different activities. In this case also there is evidently at first combination of the protein on DNA as an extended β-configuration molecule which is inactive, and the ratio between the quantity of extended β-configuration and α-configuration of the protein in the complex determines the differing total activity of the complexes. For evidence of the correctness of such an explanation of the mechanism of the observed effects, however, we must obtain a compound of DNA with protein in which the special configuration of the latter on the DNA molecule will be more or less the same: for example, to obtain a compound where the possibility would be excluded of an unfolding of the protein in the β-configuration which would assure inactivation of the protein according to our ideas. Starting from theoretical considerations, it is possible to obtain such a compound.

It is assumed that the unfolding of the protein in the β-configuration occurs on the molecule of DNA in the extended B-configuration [11] since there is then a steric relation between the nucleotides of the DNA and the amino acid residues of the protein [8].

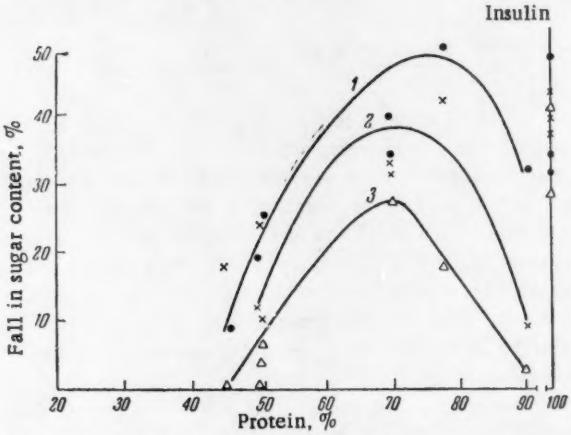

Fig. 2. Activity of insulin bound to DNA depending on its content in the complex. Amount of insulin in the experiments – 0.5 units/kg. Percent fall in blood sugar: 1) after 1 hour; 2) after 2 hours; 3) after 5 hours.

When we go from the extended to the folded configuration [12] the internucleotide distances in it are shortened which results in a change in the period of identity and a destruction of the steric relation between the dipeptides of the protein in the β-configuration and the dinucleotides of the DNA. Hence, it is possible to get artificial nucleoproteins in which the protein is combined with DNA only in the α-configuration if the DNA used for the formation of the complex occurs only in the compressed form. Such configurational changes occur in bonds of DNA with protein due to neutralization of the electronegative phosphorus groups in the stretched molecule by basic amino acid radicals of the protein and the resulting partial dehydration of the DNA molecule. The presence of the shift in configuration is easy to observe by the decrease characteristic viscosity of the DNA in the compound with the protein which depends on the decreased asymmetry of its molecule. We have tried to obtain the configuration of DNA in another way, namely, by partial neutralization of the phosphors groups in the molecule by simple and complex amines.

Obtaining Compounds of DNA with Amines

For the study of compounds of DNA with amines we used methylamine and a base which is an amino derivative

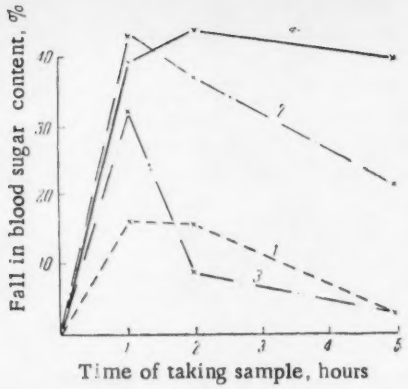

Fig. 3. Comparative activity of free insulin and insulin bound with DNA depending on time of action. DNA-insulin complex with protein content: 1) 44-50%; 2) 69-77%; 3) 90%; 4) insulin. We give average data from four experiments.

of tetramethoxydibenzocycloheptatriene, or colchamine ($C_{21}H_{25}O_5N$). The latter compound was used to study the effect of a structure of an amine radical on the properties of the resulting compound.

The DNA-amine complexes were obtained in a citrate buffer, pH 6.5, ionic strength 0.15. To a solution of the amine hydrochloride in the buffer we added gelatinous DNA (precipitated from alcohol) with different relative amounts of DNA and amine. The resulting complex was separated from unreacted amine by long dialysis against physiological saline solution. In another variant of the experiment the resulting complex was precipitated from the reaction mixture by ethyl alcohol (two volumes). In these compounds we determined N, P, and the characteristic viscosity of the solutions.

The results of the experiments are given in Table 1.

As the results show, DNA combines with amines in different ratios. The amount of bound amine increases with rise in starting ratio of DNA/amine to a definite limit which corresponds to maximum saturation of phosphate groups in the DNA molecule by the amine. When somewhat more than half the phosphate groups in the molecule are neutralized, there occurs a compression in DNA structure shown by the decrease in the characteristic viscosity of its solutions. Table 2 gives the comparative size of the change in viscosity of the compounds of DNA with amines and proteins.

The experiments showed that the ratio of the resulting viscosity to the initial viscosity, which reflects the degree of change in asymmetry of DNA in the compound, does not depend on the structure of the amine radical and is identical with the coefficient of change in viscosity of the compounds of DNA with different proteins. In all cases the viscosity of DNA in the compounds is about half the viscosity of the starting DNA. In the former report, the characteristic viscosity of natural deoxyribonucleoproteins was also half the viscosity of the corresponding DNA [13]. The results of these experiments showed that the degree of compression of DNA in the different compounds was not specific and did not depend on factors which produce this compression of the molecule that corresponds to the most stable configurational state of DNA in such compounds. From the calculations which were made [12], DNA in compounds with proteins is shortened 30% (which corresponds to a degree of shortening of DNA on transformation from the paracrystalline B to the crystalline A-configuration, also 30%). According to the results, the same degree of shortening can be produced not only by compounds with proteins, but also by other factors, namely, by combination of DNA with low molecular weight bases of the type of simple and complex amines.

Compounds of DNA with amines can serve therefore as starting substances for obtaining DNA compounds in the compressed form with proteins in the α-configuration, since the possibility of combination of proteins with the compressed form of DNA in the extended β-configuration of the molecule is obviously excluded because of the steric lack of correspondence of these structural forms.

As a model we obtained the compound DNA-methylamine-chymotrypsin (DNA-MA-chymotrypsin).

DNA-MA-Chymotrypsin

To obtain the complex, we used the compound of DNA with methylamine which contained the minimum number of molecules of the base required to carry out the transformation of DNA from the extended to the compressed modification. This condition, as experiments showed, was obtained by compounds with above 50% neutralization and the corresponding quantity of free phosphate groups. The presence of the latter is an important condition, since the basic type of bond of DNA with proteins in the α-configuration (and also in the β-configuration) is evidently a salt bond between the phosphate groups of DNA and the basic amino acid radicals of the protein. Actually, as special experiments showed, DNA fully saturated by amines does not give compounds with proteins.

The DNA-MA-chymotrypsin complex was obtained by mixing solutions of α-chymotrypsin in a citrate buffer, pH 4.5, ionic strength 0.15, with solutions of DNA-MA in 0.14 M NaCl at a ratio of DNA : protein = 1 : 2 to 1 : 5. The resulting complex was separated from the solution by precipitation with ethyl alcohol (two volumes), washed with 70% alcohol, and dissolved in 1 N NaCl. In another variant of the experiment the DNA-MA compound was first precipitated with two volumes of alcohol and was added to a gel to the solution of protein in the citrate buffer; in this case the complex was isolated directly in the insoluble state. The precipitate was separated by centrifuging, washed from the starting substances and dissolved in 1 M NaCl (all the work on obtaining the complex was carried out at 2-4°). In the solution we determined the DNA content by phosphorus, the protein by nitrogen (in the calculation we took account of N in the DNA-MA molecule), and the specific proteolytic activity of the

TABLE 1. Physicochemical Characteristics of the DNA-Amine Complexes

Complex	Starting ratio DNA/amine	Composition of complex		Amt. of neutralization of PO_4 groups by amine, %	[η] 100 ml/g	
		DNA mg/ml	amine, mg/ml		initial DNA	compound DNA-amine
DNA-methyl-amine	1:0,12	0,245	0,010	44	32,1	33
» »	1:0,20	0,30	0,0162	58	31	16,3
» »	1:1	0,192	0,0185	102	31	17,3
» »	1:2	0,293	0,026	94	32,1	16,8
DNA-colchamine	1:1	0,231	0,088	34	31,1	31,7
» »	1:5	0,163	0,182	99	31,1	15
» »	1:7,5	0,154	0,185	106	31,1	14,7

chymotrypsin in the complex compared to the activity of an equivalent amount of free enzyme. The results of the experiments are given in Table 3.

As the results show, α-chymotrypsin combined with DNA occurs in the compressed configuration and shows a higher activity than free α-chymotrypsin. Hence, in compounds in which there is no possibility of combination of α-chymotrypsin in the form of the extended β-configuration of the molecule, there is not only no decrease in activity of the enzyme, but, on the contrary, there is a marked activation (by 50-70%). Thus it is shown that the earlier observed effect of inactivation of the enzyme (and evidently also for insulin) depends on the presence in the composition of these compounds of a definite amount of protein combined with DNA in the form of an extended β-configuration of the molecule. The effect of activation of the enzyme in compounds with the compressed form of DNA can also be expected from an analysis of the results of the previous experiments (Figs. 1 and 2). Actually, according to these results, the activity of α-chymotrypsin and insulin in this type of complex reaches the level of the controls (not bound to the DNA). If we consider that, in all cases of the experiments, we studied equal amounts of protein and that part of the latter was bound to DNA (as follows from the data of the analysis of complexes which contained up to 50% protein) in inactive form, then it is clear that the observed effect of getting an activity equal to that of the control can be obtained only under conditions of the presence of activated protein in the complex.

These results of the experiments indirectly confirm the presence in artificial nucleoproteins of two types of configuration of the protein molecule, each of which assures a different degree of intensity of the biological function of the protein. It is especially interesting to obtain an activated state of the protein in the α-configuration by combining it with the compressed form of DNA. This state apparently occurs as a result of partial deformation, while the specific character of the uptake of polypeptide chains of the protein and the specificity of their distribution on the DNA molecule are retained; these are determined when the complex forms by saltlike and hydrogen bonds. Unfolding of the protein molecule is accompanied by an increase in steric accessibility and

TABLE 2. Change in Characteristic Viscosity in Compounds of DNA with Amines and Proteins

Complex	(η) 100 ml/g		$[\eta_1]/[\eta_0]$
	starting DNA, η_0	DNA compound, η_1	
DNA-BSA*	44	23	0,52
» »	56	27,8	0,50
DNA-insulin	31	18	0,58
» »	31	16,6	0,53
DNA-methyl-amine	31	16,3	0,53
» »	31	17,3	0,56
DNA-colchamine	31	15	0,48
» »	31	14,7	0,48

* BSA – beef serum albumin [3].

reactivity of a number of reactive groups, and if these groups play an important part in the production of the specific activity of the molecule, then the result of this will be the intensification of the biological function of the protein.

In this connection we are interested in the recent communication [14] on the increase in proteolytic activity of trypsin and chymotrypsin under conditions of increased pressure ($2\text{-}4 \cdot 10^3$ atm), the effect of which can obviously be to bind protein molecules by configurational changes with their deformation and a tendency to occupy a minimum volume.

We should mention the effect of activation of the enzyme urease when it is bound with RNA [15], and also the increased amount of free amino and thiol groups and the rise in reactivity of the amino groups of the protein combined with RNA [16].

Our suggestion of the presence of different types of arrangement of the protein on the DNA molecule evidently remains valid also for natural desoxyribonucleoproteins (DNP). According to the results of various authors, natural DNP isolated by different methods contains 58-60% protein. According to calculations the amount of protein which can be arranged on a DNA molecule in the β-configuration is 15-25% and the rest of the protein bound to DNA must be arranged on the DNA in the α-configuration. The data on this question in the literature are contradictory: There is evidence of the presence of fully extended polypeptide chains on the DNA molecule [8], and also of the presence of protein in the molecule which is only partly unfolded [17].

TABLE 3. Change in Proteolytic Activity of α-Chymotrypsin in Compounds with DNA-Methylamine

Preparation	α-Chymotrypsin content, %	Δ proteolytic activity
DNA-MA-α-chymotrypsin	73,2	0,245
α-Chymotrypsin	—	0,159
DNA-MA-α-chymotrypsin	66,0	0,242
α-Chymotrypsin	—	0,159
DNA-MA-α-chymotrypsin	72	0,339
DNA-MA-α-chymotrypsin	72,3	0,340
α-Chymotrypsin	—	0,197

Note. In determination of the activity, we took 0.125 mg of free and bound enzyme. Incubation was carried out for 20 min.

TABLE 4. Change in Proteolytic Activity of α-Chymotrypsin in Combination with DNP

Preparation	Content, %	Δ proteolytic activity
DNP-α-chymotrypsin	65,3	0,263
DNP-α-chymotrypsin	63	0,240
α-chymotrypsin	—	0,159
DNP-α-chymotrypsin	72,3	0,272
α-chymotrypsin	—	0,197

Starting from this, it was interesting to study the possibility of activation of the protein combined with native natural nucleoproteins (DNP) previously freed from part of the protein bound in labile manner in the molecule, but retaining the compressed DNA configuration in the structure. For this purpose we obtained DNP, as far as possible native, which contained 20-25% protein. The preparations were obtained by the method of incomplete deproteinization of calf thyroid DNP (N/P = 2.1-2.2). Such preparations resembled DNA in some properties (solubility, absence of relaxation), but measurement of the degree of asymmetry of DNA in them, determined by comparison of the characteristic viscosities of the DNP solutions, showed that the DNA in these preparations occurred in the compressed configuration and the retention of 20-25% of the protein left a sufficient number of free phosphate groups to combine with and orient the structure of other protein molecules.‡ The resulting preparations were used to obtain compounds of DNP with α-chymotrypsin.

These were obtained by the method described above. Comparison of the specific proteolytic activity of α-chymotrypsin in these complexes with the activity of the free enzyme showed (Table 4) that the enzyme is activated on similar molecules of DNP and the degree of its activation is about the same as in the compounds DNA-MA.

The results of the experiments showed that the ideas given above are also correct for natural DNP and confirm our suggestion of the role of the configuration of DNA in determining the structural orientation of its compounds with protein and the relation of the molecular organization of the protein and the intensity of its biological function.

DISCUSSION

The compounds of nucleic acids with proteins give one of the most interesting examples of the presence of interconnections and interdependence in the structural organization of substances. The protein determines the configuration of DNA and, by combining with it in the form of an extended polypeptide chain in the β-configuration caused a compression of the extended structure of DNA. The change in configuration of DNA in its turn determines a new type of molecular organization of the protein which is combined with it with some deformation on the surface of its molecule.

The interdependence of the structural changes of the substances evidently also determines the dependence of the functional activity of the compound on these facts. Recently data have appeared which show that the biological activity of the nucleic acids apparently depends on a considerable degree on their structure which is determined by the proteins combined with them [18]; reports have also appeared on the change in biological activity of proteins due to unfolding of their molecules when combined with RNA [15, 16].

The study of the properties of the compounds of high molecular weight DNA with biologically active proteins, α-chymotrypsin and insulin, permits us to assume with a very great degree of probability that the protein first combined with DNA, characterized by a fully extended β-configuration of the molecule, is not active and that DNA in the extended configuration combines with a protein and inactivates that part of the protein which causes compression of its molecule. On the other hand, the DNA changed under the influence of the protein (amines can replace protein in this role) apparently in the compressed A-configuration [12] combines with the protein in the α-configuration as a partly deformed structure which has a higher functional activity than the native protein. This idea is indirectly confirmed by the experimentally established dependence of the activity of the compound on the amount of protein in the complex, that is, on the quantitative ratio of extended DNA at one or another configuration of the protein molecule, and is directly confirmed by the observation of the effect of increased activity of α-chymotrypsin when it combines with DNA in the compressed configuration (with DNA-methylamine and with partly deproteinated nucleoproteins).

‡ As experiment showed, enzymes can scarcely be added to the undeproteinated nucleoprotein.

The mechanism of inactivation of the protein when it combines with the extended form of DNA is not clear. From ideas of the steric relation between the periods of repetition of nucleotides and the intervals between the side chains in fully extended polypeptide chains [8] we could assume that all the basic amino acid residues of the peptide chain are bound with phosphate groups of DNA by direct salt forming electrolytic bonds. Thus, on the one hand, if some of the active centers of the molecule are basic amino acids, the latter will be blocked, and on the other hand, unfolding of the popypeptide chains will destroy the topography of the molecule, the specific orientation of the reactive centers in its structure. It is also possible that in the explanation of the inactivation mechanism a definite role is played by the steric inaccessibility of the protein molecule when it is distributed between the spirals of the polynucleotide chain of the DNA. There is even less material to explain the effect of raising the activity of the protein in the α-configuration in the deformed state on the DNA structure.

In the experiments on the study of the biological activity of compounds of DNA and α-chymotrypsin or insulin, when the complexes contained more than 75% of the latter, we find a decrease in the functional activity of the protein. To explain the mechanism of this observation we have expressed the opinion that in the inactivation of the protein in such complexes a great role is obviously played by the intermolecular bonds which with increasing content of protein block the reactive centers of the molecules and decrease the flexibility (elasticity) of the system.

In our earlier studies of the activity as related to phosphatases as the enzymes bound to DNA [5] we showed that the enzyme activity in the complex depended on the contractile properties of the system. Under the influence of factors which decreased the elasticity of the complex, the enzymatic activity of the protein was also decreased. In the study of dependence of the contractile properties of the compounds of DNA-α-chymotrypsin on their protein content [1] it was shown that over a definite range of protein content in the complex (63-70%) the elasticity of the latter, expressed as the size of the relative relaxation, was a maximum and occurred at about a single level; when the amount of protein increased to 77-80% of the contractile properties of the complex decreased markedly, falling to zero at 94% protein in the complex, which obviously corresponded to a state of complete blocking of the bonds in the molecule due to increased intermolecular reactions. The data on the study of dependence of enzyme (or hormonal) activity on the amount of protein in the complex show the presence of the same regularities, and the ranges of protein content which correspond to the maximum and decreased values of contractile properties and activity of the compounds almost agree. Such a relation of properties seems regular, but the explanations offerred cannot be considered sufficient and require further experimental studies.

Thus, the experimental data which have been presented confirm the nonhomogeneous character of the structural organization of the protein on the DNA molecule and the dependence of biological activity of the compounds on these factors. The similarity of the change in intensity of biological function of the proteins of two different systems, under experimental conditions in vitro and in vivo, indicate the possibility of the generality of the effect. The same phenomenon of increased enzyme activity when it is combined with an artificially obtained compressed form of DNA (in DNA-amines) and in natural DNP (with a naturally compressed form of DNA), and also the observation of the ability of artificial nucleoproteins to be included in the metabolic reactions of the organism show the presence of a general structure in these compounds in common with the natural nucleoproteins. Evidently this structural generality has a relation or even assures a similar character to the structural changes of DNA when it passes from the extended to the compressed configuration under the influence of various agents; this is shown in our findings of the similarity of changes in characteristic viscosities of compounds of DNA with different proteins and amines and also (according to the literature data [13]) of natural nucleoproteins as compared to the viscosity of the starting DNA.

SUMMARY

We have shown the presence of a definite dependence of activity of α-chymotrypsin and insulin bound with DNA on the content of the protein in the complex expressed (similarly for both systems studied) in inactivation of the protein when it is present in amounts less than 50%, in increase in activity of the control level at 70-75% protein, and then fall in activity with further rise in protein content.

We have showed that when DNA reacts with amines, at a definite ratio of components there is a transformation from the extended to the compressed form of DNA and the degree of compression of DNA which determines the change in characteristic viscosity, is the same in complexes with different amines as with protein (and also in natural nucleoproteins).

We have established an increase in proteolytic activity of chymotrypsin in compounds with DNA in the compressed configuration (with DNA-amines and with incompletely deproteinated nucleoproteins).

From these data we believe that the intensity of biological function in protein bound to DNA depends on its structural organization which gives a different character of unfolding of the protein molecule in combinations with DNA in the extended or compressed configurations.

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DIFFERENT METABOLIC PATHWAYS OF C^{14}-RIBONUCLEOSIDES IN TUMOR AND NORMAL CELLS

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Translated from Biokhimiya Vol. 25, No. 1, pp. 55-60, January-February, 1960

Original article submitted June 2, 1959

The elucidation of biochemical differences between normal and tumor cells appears to be a prerequisite for the development of rational methods for the chemotherapy of cancer.

It is clear that the whole series of chemotherapeutic agents affect different parts of the nucleic metabolism of cells [1-7]. Therefore, a detailed study of the mechanisms of nucleic acid biosynthesis in normal and tumor tissues will make it experimentally feasible to find the active group whose suppression will harm a tumor cell more than a normal one.

It has been established by the work of foreign investigators that tumors differ from normal tissues in their ability to use various labeled compounds as precursors in the synthesis of nucleic acids [8-15]. In similar investigations, much attention has been given to working out the metabolism of ribonucleosides and their mono-, di-, and triphosphoric esters. They appear to be intermediate products in the synthesis of RNA and DNA [16, 17, 18], and, besides this, perform a variety of functions in the cell as coenzymes in a series of respiratory and synthetic processes [19-22], and may themselves undergo oxidative changes.

The degree to which ribonucleosides and their derivatives are used for the synthesis of nucleic acids in cells of normal tissues and tumors depends, evidently, on the activity and type of reactions in which they are involved, either as substrates or coenzymes.

An attempt was made in the present work to establish the relation between just two of the possible metabolic pathways of ribonucleosides in normal and tumor cells, these being: their use as substrates for the synthesis of RNA and DNA, and the oxidative transformation of their carbohydrate component.

With this aim in mind, we incubated a suspension of normal and tumor cells in vitro in the presence of uniformly labeled C^{14}-ribonucleosides, and then determined the relative distribution of label between RNA, DNA, and $C^{14}O_2$ but also tried to identify the intermediary products of the oxidation of ribose by means of paper chromatography and radioautography.

Incorporation of C^{14}-Ribonucleosides into Nucleic Acids, and Oxidation to $C^{14}O_2$ in Liver Cells and Ascitic Tumors Incubated In Vitro

Cell suspension	Incubation period	Concentration of nucleic acids, mg/g dry tissue		Radioactivity					
				RNA		DNA		$C^{14}O_2$	
		RNA	DNA	counts/min · mg RNA	% original activity	counts/min · mg RNA	% original activity	counts/min · mg RNA	% original activity
Liver	0	8.38 ±0.15	3.0 ±0.1						
	30 min	7.75 ±0.11	2.9 ±0.1	0	0	0	0	3600±366	2.8
	1 hr	7.10 ± 0.14	2.7 ±0.05	0	0	0	0	5100±250	4.0
	2 hr	6.8 ±0.1	2.7 ±0.05	0	0	0	0	8200±700	6.4
	3 hr	7.2	2.4	139	0.13	0	0	10,300	8.0
Ascitic hepatoma	0	3.96 ±0.08	6.2 ±0.1						
	30 min	4.16 ±0.11	6.25 ±0.25	199 ±55	1.03	30.8 ±2.8	0.25	349±7.0	0.43
	1 hr	4.27 ±0.05	6.7 ±0.05	215 ±54	1.13	33.4 ±10.6	0.26	628±76	0.82
	2 hr	4.27 ±0.15	6.5 ±1.0	247 ± 35	1.40	53.6 ±5.1	0.45	1173±133	1.45
Erlich ascitic carcinoma	0	4.2 ±0.1	4.25 ±0.15						
	30 min	4.5 ±0.15	4.32 ±0.1	215 ±68	1.13	50.9 ±1.3	0.23	903±140	0.9
	1 hr	4.5	4.50 ±0.13	273 ±107	1.30	75.7 ±16.2	0.37	1320±241	1.35
	2 hr	4.51 ±0.16	4.72 ±0.05	186 ±10	0.8	54.0 ±1.1	0.25	1840±66	1.80

MATERIALS AND METHODS OF INVESTIGATION

Experimental materials – tumor explants: rat ascitic hepatoma (Zazhdel' strain), and Erlich ascitic carcinoma. Rat liver was used as a control (normal tissue).

Preparation of C^{14}-Labeled Ribonucleosides

We obtained ribonucleosides completely labeled with C^{14} by allowing a suspension of the single-celled alga (Lagerheimia ciliata to photosynthesize in an atmosphere of $C^{14}O_2$.*

Composition of Beneke's culture solution: 0.1 g $MgSO_4$; 5 g $Ca(NO_3)_2$; 0.2 g K_2HPO_4; 3 mg ferric citrate and citrate acid; 1 liter boiled tap water.

A small volume of algal suspension (about 1 million cells) was transformed to 10 liters of culture solution. Under these conditions, it was possible to get about 20 g dry weight of cells in 1 month.

Before beginning the photosynthesis experiment, we separated the cells from the nutrient solution by centrifugation, and suspended them in a small volume of Beneke's solution to give an estimated 15 g dry weight of cells in 1 liter of solution. This suspension was poured into a thick-walled bottle (5-7 liter capacity), which served as a photosynthesis chamber. In the mouth of the bottle we put a stopper with two glass tubes. Onto one of the tubes was attached a cup containing two millicuries of $BaC^{14}O_3$, and stoppered the bottle. We first evacuated the bottle slightly, then sealed it hermetically, and poured 1 ml 6 N $HClO_4$ into the cup containing the $BaC^{14}O_3$ to bring about the evolution of $C^{14}O_2$. Photosynthesis was continued for 72 hours at room temperature, in bright light, and with vigorous shaking (in a shaker). At the end of the incubation period, 3 ml of 5 N NaOH was introduced into the cup containing $BaC^{14}O_3$, and the bottle was shaken for an hour to absorb any remaining $C^{14}O_2$.

We obtained preparations of the C^{14}-RNA and then of the ribonucleotides by treating the algae according to the method of Rose and Schweigut. The cells were separated from the nutrient solution by centrifugation. The pigments and lipids were carefully extracted by means of hot acetone, alcohol and ether, and ether. The acid-soluble substances were extracted in the cold with 5% TCA.

We carried out the alkaline hydrolysis with 0.5 N KOH for 24 hours at room temperature, and the ribonucleotides were precipitated after separation from the DNA and proteins in the form of barium salts. Barium ions were removed as $BaSO_4$, and the solution contained a mixture of ribonucleotides uniformly labeled with C^{14}. At this stage, we carefully freed the solution from protein impurities by means of repeated precipitation with alcohol in acid solution. The alcohol was removed by vacuum distillation, and the dry residue consisting of ribonucleotides was dissolved in water.

Since, according to the literature, ribonucleotides are reported to diffuse only slightly through a cell membrane [13], we subjected the mixture of C^{14}-ribonucleotides to the action of alkaline phosphomonoesterase, obtained from the small intestine of the rat, according to the method of Doman and Kagan [24]. To the solution of C^{14}-ribonucleotides in ammonium-acetate buffer at pH 9.6 was added the preparation of phosphatase to give 2 mg of enzyme per 5-7 mg of the ribonucleotides, and $MgSO_4$ to a final concentration of 0.013 M. The mixture was floated with toluene, and incubated for 72 hours at 37°. About 90% of the ribonucleotides were broken down to ribonucleosides by this treatment. At the end of the incubation period, the protein was carefully precipitated with $HClO_4$ and alcohol; the $HClO_4$ was removed by the addition of KOH, and the solution of ribonucleosides was evaporated to dryness, and then dissolved in water. The yield of C^{14}- ribonucleosides from 15 g of algae varied in different experiments from 6 to 13 mg, with a specific activity of 3.5×10^3-3.8×10^4 counts/min/μM C.

When separated by paper chromatography with a mixture of isopropanol, ethanol and 5 N HCl in the ratio of 6:2:2, the mixture of ribonucleosides gave 4 spots: R_{f_1} 0.25-0.30; R_{f_2} 0.38-0.42; R_{f_3} 0.47-0.53; R_{f_4} 0.64-0.66 corresponding to guanosine, adenosine, cytidine, and uridine respectively (Fig. 1).

Judging by the extreme faintness of the guanosine spot, and, to a lesser extent, of cytidine, it is possible that these nucleosides were partially deaminated during the incubation with intestinal phosphatase, which clearly contains a considerable admixture of deaminases. The radioautograph of the chromatogram on which the C^{14}-ribonucleosides were run is shown in Fig. 1.

This mixture of C^{14}-ribonucleosides was used by us in experiments in vitro for the purpose of studying the different pathways of metabolism of these compounds in tumor and liver cells.

Design of In Vitro Experiments

For our work with the ascitic tumor forms we separated the tumor cells from the ascitic fluid and erythrocytes by centrifugation in the cold at 300-400 rpm for 4-5 minutes; washed them in Krebs-Ringer-phosphate buffer (pH 7.2); centrifuged them for 5 minutes at 2000 rpm; suspended the cells, and prepared a 20% suspension in the same buffer.

We prepared a suspension of liver cells by carefully grinding tissue for 2 to 3 minutes in three to four times its volume of Krebs-Ringer-phosphate buffer, using a hand-operated glass homogenizer, separated the cells from fragments of protoplasm, nuclei, and erythrocytes by centrifugation at 400 rpm, and then prepared a 20% suspension in Krebs-Ringer-phosphate buffer. The 20% cell suspension was usually incubated with the mixture of C^{14}-ribo-

*The culture of Lagerheimia ciliata was kindly given to us by N. S. Gaevskaya, managing director of Hydrobiology at the Institute of Fisheries, from where we also learned the method of culturing the algae in Beneke's mineral medium, for which we use this opportunity to convey our appreciation to N. S. Gaevskaya and N. N. Smirnov.

nucleosides (20,000 counts/min in 0.1 ml solution), Mg ions (0.013 M), glucose (5 mg), nicotinamide (1 mg) (total volume of the mixture 1.7-2.0 ml) at 37°, for 30 to 180 minutes in air, using a Warburg apparatus. In the center well we put filter paper that had been soaked in 0.2 ml 5 N KOH, and in the side arm, 0.3 ml 2 N $HClO_4$. The apparatus was hermetically sealed with stoppers. At the end of the incubation period the $HClO_4$ solution was poured out of the side arm onto the incubation mixture, and the apparatus was maintained in the thermostat for a further 30 minutes to ensure complete absorption of the $C^{14}O_2$ by the alkali. In the controls, we poured a solution of $HClO_4$ onto a suspension of cells in the apparatus. At the end of the incubation period, the $C^{14}O_2$ absorbed by the KOH from the filter paper was eluted with water, and the $BaC^{14}O_3$ was precipitated in water by adding an excess of 5 N $BaCl_2$.

The suspension was filtered under low vacuum through a close-textured filter paper; the residue was dried at 37° and suspended; the radioactivity of the $BaC^{14}O_3$ was then determined by means of a window counter. We corrected for self-absorption. The contents of the vessels were quantitatively transferred to centrifuge tubes. To obtain complete precipitation of the proteins in the tubes, we added sufficient 5 N $HClO_4$ to give a final concentration of 0.4 N, carefully cooled them, and centrifuged them in the cold. The centrifugate contained the main bulk of the acid-soluble substances.

We extracted the remainder of the acid-soluble substances and lipoids from the precipitated cells according to Schneider's method [25]. The RNA was hydrolyzed to its ribonucleotides by treatment with 0.5 N KOH for 18 hours at 37°. In order to precipitate proteins and DNA, the hydrolyzate was acidified with 5 N $HClO_4$ to pH 1.0. The centrifugate was neutralized with KOH, freed of the $KClO_4$ and the solution was brought to a determined volume (2-4 ml); the amount of RNA was then determined according to Mejbaum [26], and its radioactivity (0.25-0.5 ml of the neutralized hydrolyzate per target) measured with the help of a window counter. DNA was extracted by two successive treatments with 1 N $HClO_4$ at 80°, and freed from $HClO_4$ by the addition of KOH to a pH of 7.0; the amount of DNA was determined (spectrophotometrically) and its radioactivity measured.

Desalting of the Protein Free Filtrates

The protein-free filtrates were separated from $HClO_4$ by adding a solution of KOH until the neutralization point was reached; in order to determine the radioactivity 0.05 ml was taken from the 4 ml. We then proceeded to desalt them using a cation exchange column CDV-3 (SO_3H – a form analogous to Dowex-50) according to Kit's method [27]. The column was loaded with 1% HCl, and then washed with water until there was no further reaction to chloride; 4 ml of protein-free filtrate was acidified with HCl until the pH was 1, and transferred to the cation exchange (10 × 120 mm). We allowed the column to run at 0.3 ml/min. Organic acids and part of the ribonucleosides were eluted from the column with 10-15 ml 0.1 N HCl; cations and amino acids, with the exception of dicarbonic acids, remained on the resin.

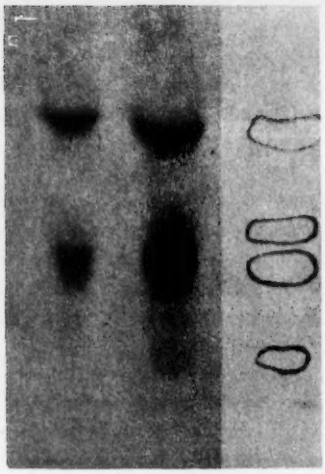

Fig. 1. Radioautograph of the chromatogram of the mixture of C^{14}-ribonucleosides. Solvent system; isopropanol – ethanol – 5 N HCl (6:2:2 by volume). On the right: diagram of the chromatogram.

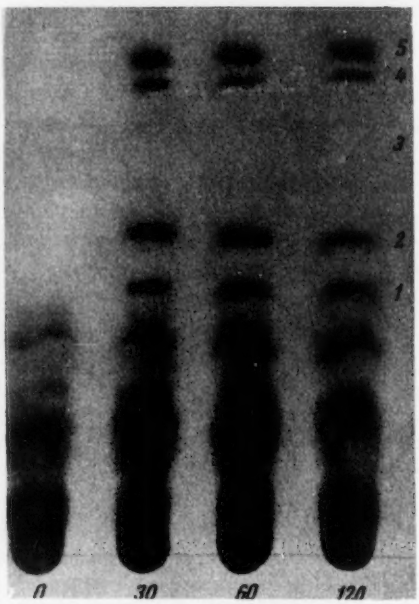

Fig. 2. Radioautographs of the chromatograms of the acid-soluble fraction of liver, before and after incubation of a mixture of C^{14}-ribonucleosides. Solvents: n-butanol, saturated with 15% formic acid. 1) Citric acid; 2) α-ketoglutaric acid; 3) lactic acid; 4) fumaric acid; 5) aconitic acid. Numbers below are incubation periods (minutes).

The HCl-eluate was evaporated to dryness in a vacuum at 27°, and the residue dissolved in 0.4 ml of water; this solution was used for the paper chromatographic separation of the C^{14}-ribonucleosides and organic acids. n-Butanol, saturated with 15% formic acid, seemed to be the most suitable solvent [28]. When ascending chromatography is used with this mixture, all the ribonucleosides remain on the lower third of the paper, while the organic acids occupy the upper two thirds. Thus, almost complete separation of the two groups of substances is obtained. We detected the ribonucleosides and their derivatives on the chromatogram by means of an ultrachemiscope, then dried the paper at 90°, and revealed the organic acids with brom cresol green (40 ml in 100 ml of ethanol).

The following are the R_f values for the organic acids: citric acid 0.33-0.37; malic acid 0.47-0.48; oxalic acid 0.50-0.52; α-ketoglutaric acid gave two spots, one at R_f 0.53-0.57, and the second at 0.75; lactic acid 0.71-0.72; succinic acid 0.75-0.76; fumaric acid 0.85-0.88. Usually on the chromatograms, the organic acids showed up as diffuse yellow spots on a blue background, and only lactic and succinic acids gave clear spots.

Since we were interested in the fate of the ribonucleosides not only as precursors of RNA and DNA, but also as possible respiratory substrates, we attempted to determine the incorporation of label into lactic acid and the components of the Krebs cycle. It was with this aim in mind that the radioautographic method was used. A radioautograph of the chromatogram of the protein-free filtrate was prepared by exposing it for a two month period in contact with Agfa x-ray film.

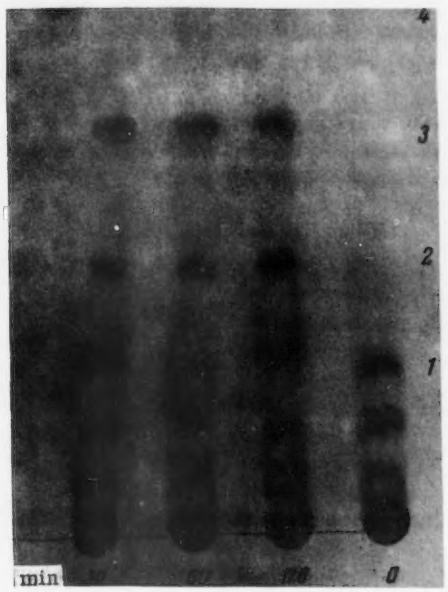

Fig. 3. Radioautographs of the chromatograms of the acid-soluble fraction of rat hepatoma (Zazhdel' strain) before and after incubation with a mixture of C^{14}-ribonucleosides. Conditions the same as in Fig. 2.

RESULTS AND DISCUSSION

The analysis of the experimental results supports the conclusion that in liver cells (as compared with tumor cells), the metabolism of the ribonucleosides lies strongly in the direction of their oxidation. Actually, we did not observe the incorporation of C^{14}- ribonucleosides into either RNA or DNA. Only in one experiment, after a 3-hour incubation, did we succeed in showing a small incorporation into the RNA fraction. The amount of both nucleic acids in the liver cells in vitro drops during the incubation period. At the same time, a rapid oxidation of the C^{14}-ribonucleosides is observed, and over a 3 hour incubation period, approximately 10% of the substrate added is found to be oxidized to $C^{14}O_2$ (Table).

In the radioautographs of the protein-free filtrates (Fig. 2), it is possible to observe a distinct incorporation of the label into the components of the Krebs cycle: citric, α-ketoglutaric, and fumaric acids, and also a small incorporation into lactic acid. It seems that the results demonstrate that, in liver cells, a considerable part of the ribonucleosides react with the nucleoside phosphorylase, which breaks them down to ribose-1-phosphate and nitrogen base.

Ribose-1-phosphate is transformed by phosphoribomutase to ribose-5-phosphate, and then, broken down by transketolase to 3C- and 2C-fragments, it undergoes further oxidative changes in the Krebs cycle.

The fate of the C^{14}-ribonucleosides in tumor cells is quite different (table, Fig. 3). Here the balance shifts towards the pathway of nucleic acid synthesis, this being shown especially by the increase in concentration of RNA and DNA over the incubation period in tumor cells exposed to C^{14}-ribonucleosides, but also by the incorporation of label into both the nucleic acids. It is true that this incorporation is not great, being 1-2% of RNA and 0.2-0.4% for DNA of the original activity. This can be explained by the fact that ribonucleosides, before they can become components of the nucleic acids, must go through a series of transformations in the cell, resulting in the formation of ribonucleoside-diphosphates and deoxyribonucleoside-triphosphates – the immediate precursors for the synthesis of RNA and DNA.

The rate of oxidation of C^{14}-ribonucleosides to $C^{14}O_2$ in ascitic hepatoma and Erlich carcinoma cells was one fifth that of the liver cells, while on the radioautographs we found lactic acid to be the main intermediary product of the oxidation (Fig. 3). The incorporation of label into the components of the Krebs cycle proceeded less actively than in liver cells.

Evidently, in the tumor cell, ribose-phosphate derived from ribonucleosides is, as in liver cells, first acted on by transketolase, but its further oxidation proceeds for the most part via the glycolytic pathway, and only partly through the Krebs cycle. The fate of lactic acid and other products of C^{14}-ribonucleoside oxidation in tumor cells has not been investigated by us. However, from the main body of experimental evidence [9], one may surmise that

they are not only utilized in the synthesis of ribose and desoxyribose, but are also involved in transamination reactions in amino acid and protein metabolism.

Thus, not only are the ribonucleosides of tumor cells used, in the first place, as building blocks for the synthesis of the nucleic acids, but also the products of their partial oxidation are again involved in metabolism as substrates for a whole series of reductive resyntheses. This ability of tumor cells to use intermediary products of metabolism for the synthesis of high-polymer compounds would seem to also be the basis for their rapid growth and division.

SUMMARY

Upon incubation of a cell suspension of rat ascitic hepatoma and of Ehrlich's ascitic carcinoma in a medium containing ribonucleosides fully labeled with C^{14}, the latter are utilized for synthesis of both RNA and DNA. Only a small part of the C^{14}-ribonucleosides is oxidized by tumor cells to $C^{14}O_2$, mainly via aerobic glycolysis (accumulation of the label in lactic acid). Under the same conditions hepatic cells exhibit only an insignificant incorporation of C^{14}-ribonucleosides into RNA and no incorporation whatsoever into DNA, but show a high rate of oxidation of the carbohydrate component of the nucleosides to $C^{14}O_2$. The intermediate oxidation products are represented by the components of the Krebs cycle, viz., citric, α-ketoglutaric and fumaric acid.

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THE ROLE OF DISULFIDE AND METHYL GROUPS IN THE PROCESS OF AGING

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Translated from Biokhimiya Vol. 25, No. 1, pp. 61-67, January-February, 1960

Original article submitted May 21, 1959

While the problem of aging has been the subject of study since early antiquity, in recent times special attention is being devoted to the investigation of the biochemical factors in the processes of aging, where the changes in metabolic processes could assist in finding new ways in the prophylaxis and therapy of aging.

At the present stage of our scientific knowledge there is much information available which deals with the biochemical changes in the animal organism in relation to age [1-10], most of which is concerned with the role played by protein.

Having started in our earlier studies [11-14] from the assumption that the SH groups play an important role in the processes of oxidation-reduction and detoxication, we investigated in rats the changes in amino acid and glutathione contents with age, as well as the feasibility of changing the concentration of these substances by the administration of methionine and cysteine. The results showed that in old rats the methionine concentration in blood serum is lowered, while that of cystine and oxidized glutathionine is increased; the administration of methionine and cysteine resulted in a lowering of the concentration of cystine and of oxidized glutathione, with an increase in methionine and cysteine. The present work is an extension of these studies.

Studies were undertaken on the dynamics of thioamino acids levels in old animals as affected by the administration of cysteine, methionine, serine, novocaine, dicaine and para-aminobenzoic acid (PABA), as well as the dynamics of aspartic and glutamic acids as affected by cysteine. The reasons for such a setup were the following:

1) The administration of cysteine and methionine to old animals normalizes the contents of these substances in the blood;

2) Serine participates in the synthesis of homocystine, and is thus capable of influencing the formation of cysteine;

3) Novocaine stimulates the methylation process via PABA [15] which is liberated in the organism as a result of novocain hydrolysis [16]. As to dicaine (n-butylaminobenzoate dimethylaminoethanol hydrochloride) since it is similar to novocain in its effect, it does not liberate any PABA in the organism, and thus becomes a convenient control substance for the deciphering of the mechanism of novocaine action. The administration of PABA can thus permit the differentiation between the action of PABA which is liberated in the organism from novocaine, and the PABA introduced as such, which our earlier data seem to have indicated.

The amount of aspartic acid is of interest in view of its close association with the metabolism of thioamino acids via homoserine synthesis.

METHODS

The experiments were conducted on old animals of various species – dogs, rats, rabbits, guinea pigs – the number of which are given below. The substances under these study were administered daily to the animals of the experimental group, the duration of the experiments being from 16 to 30 days.

Methionine, cysteine, and serine were given per os in amounts of 50 mg/kg; PABA was given intramuscularly, 10 mg/kg; novocaine and dicaine, were given intramuscularly, 5 mg/kg. The determination of methionine was done by the method of Hess and Sullivan [18], that of cysteine and cystine, by the polarographic method of Brdicka [19, 20]. Aspartic and glutamic acids were determined by the ascending chromatography method of Mathias, using a kaolin column, the analysis itself being conducted according to Fromageot and Jutisz [21]. All the determinations were done immediately after the end of the experimental period.

RESULTS

The Effect of the Compounds Tested on the Thioamino Acid Contents in the Blood Serum and Organs of Old Animals

Methionine and cysteine. 480 old animals (rats, guinea pigs, rabbits and dogs) were divided into three groups: the controls, those receiving methionine, and the ones receiving cysteine. The duration of the experiment was 16 days.

As shown by the experimental results, the thioamino acid content in the blood plasma of the experimental animals changes consistently; the administration of methionine brings about an increase in cysteine, while cysteine causes an increase in methionine (Fig. 1).

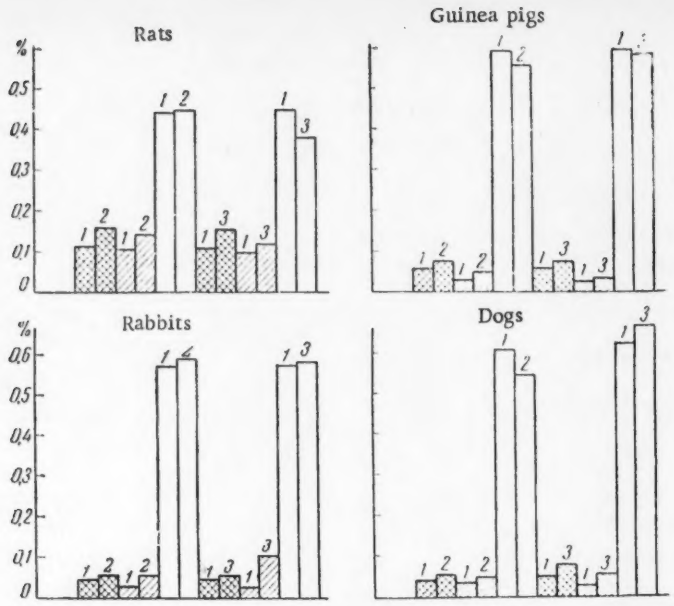

Fig. 1. Effect of methionine and cysteine on the thioamino acid contents in the blood serum of old animals.

Serine. 48 old rats were divided into two groups: the controls, and the ones having received serine for a period of 16 days. The determination of thioamino acids in the blood serum showed a reduction in the methionine and cystine content, and an increase in cysteine (Fig. 2).

Novocaine and dicaine. Ninety old rats were divided into three groups: the controls, those receiving novocain, and those receiving dicaine. Novocaine, as well as dicaine, was administered for a period of 30 days. The amount of thioamino acids was determined in the liver, kidneys, spleen, lungs, blood serum and in whole blood. The results obtained (Fig. 3) show that novocaine causes an increase in methionine in the spleen, and of cysteine in the liver and kidneys. These changes in the organs are not reflected in the thioamino acid contents of the blood serum.

Para-aminobenzoic acid. Sixty old rats were divided into two groups: the controls and the ones receiving PABA. Its sodium salt was administered for a period of 21 days. This compound caused a lowering in methionine and an increase in cystine in the blood serum of old animals (Fig. 4). A comparison of these data with the effect of novocaine administration indicates a difference in the mechanism of PABA action when introduced into the organism as such, as compared with the one liberated in the organism by novocaine hydrolysis.

Effect of Cysteine on the Aspartic and Glutamic Acid Contents of Blood Serum in Old Rats

In each experiment 18 young and 36 old rats were used. They were divided into two groups: the controls, and the ones receiving cysteine for a period of 21 days. As can be seen from the results, the old animals contained 30% less aspartic acid than the young. As a result of cysteine administration, the aspartic acid level in old rats tends to rise significantly, coming close to the one observed in young animals (Fig. 5A). The glutamic acid content in the blood serum of old rats was 23.5% higher than in young ones, coming down after cysteine administration (Fig. 5B). Thus, the administration of cysteine to old animals tends to restore the normal ratio of the concentrations of aspartic and glutamic acids in the blood serum.

DISCUSSION OF RESULTS

The experiments showed changes in methionine, cystine, and oxidized glutathione content as related to age. The blood serum of old animals (rats) showed a lower methionine and higher cystine and oxidized glutathione as compared with young ones. Daily administration of methionine or cysteine to old animals of various species (rats, guinea pigs, rabbits, dogs) caused a marked increase in methionine and cysteine, and a lowering of cystine. The obtained data indicated that in old animals there is a breakdown in the mechanism: methionine → homocysteine → cystathionine, which leads to the formation of L-cysteine and homoserine (as a result of decomposition of cystathionine [22]), namely, that the biochemical equilibrium cysteine ⇌ cystine is shifted toward a more intensive formation of cystine, which leads to a lowering of methionine content. Thus, the same substance undergoes various metabolic changes depending on the age of the animal.

In old animals, the reduction of cystine to cysteine, caused by an artificially created surplus of sulfhydryl

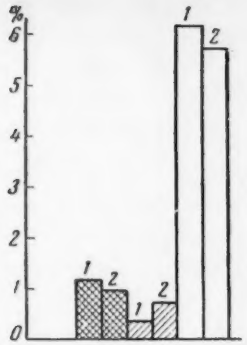

Fig. 2. Effect of serine on the content of thioamino acids in blood serum of old rats. The cross-hatched columns represent methionine, the diagonally hatched, cysteine, the blanks, cystine. 1) Controls; 2) on serine (duration, 16 days).

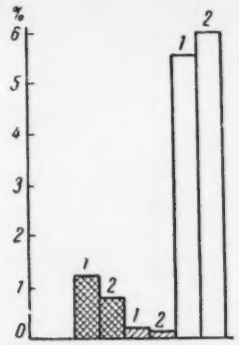

Fig. 4. Effect of para-aminobenzoic acid on the thioamino acid content in blood serum of old rats. The cross-hatched columns represent methionine, the diagonally hatched, cysteine, the blanks, cystine. 1) Controls; 2) on para-aminobenzoic acid (duration, 21 days).

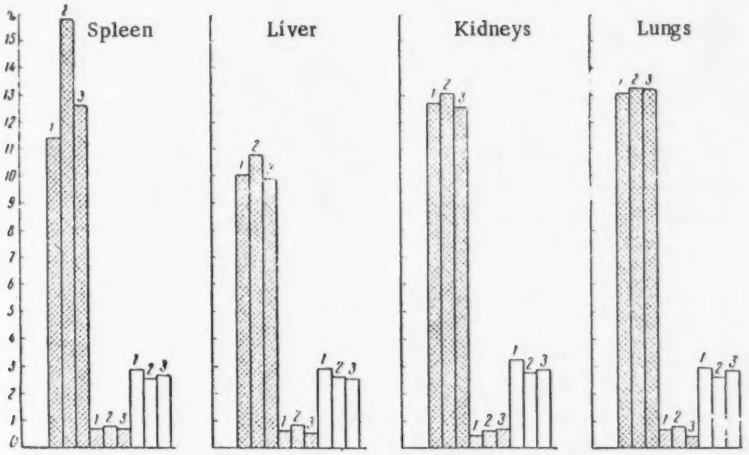

Fig. 3. The effect of novocaine and dicaine on the thioamino acid contents in the organs of old rats. The cross-hatched columns represent methionine, the diagonally-hatched, cysteine, the blanks, cystine. 1) Controls; 2) on novocain; 3) on dicaine (duration, 30 days).

groups (by the administration of cysteine). with a subsequent conversion of cysteine to methionine [23-25] represents, evidently, a reverse process as compared to the one observed in bacteria and fungi. Such would not take place in a young organism, since under these conditions cystathionine would break up into homocysteine and methionine. The fact that the administration of cysteine to old animals brings about an elevation in methionine content, the blood level of which approximates that of young animals, indicates that here the above-mentioned serves of reduction and conversion in occurring. On the other hand, taking into consideration the fact that aspartic acid participates in the synthesis of homoserine, which produces homocysteine and methionine via cystathionine, one may expect less aspartic acid in old animals than in young ones, since, as already stated, the latter are lacking the mechanism to convert cysteine to methionine. This assumption is supported by our findings which show that the aspartic acid content is lower in the blood serum of old rats as compared with young ones, and rises after a prolonged administration of cysteine. The higher level of glutamic acid in old rats is also normalized by cysteine. The indicated tendency of cysteine to reestablish the glutamic acid equilibrium undoubtedly indicates the participation of glutamic acid in the thioamino acid metabolism.

It should be also mentioned that the methionine content in the spleen of old animals increased under the effect of novocaine, namely, by the PABA which is liberated from it in the organism via hydrolysis; however, the ampli-

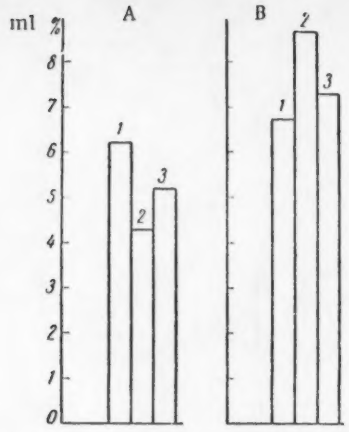

Fig. 5. The effect of cysteine on the aspartic and glutamic acid contents in the blood serum of old rats. A) Aspartic acid; B) glutamic acid. 1) Young rats; 2) old control rats; 3) old rats receiving cysteine (duration, 21 days).

tude of such a stimulation is not great enough to affect the methionine content of blood serum.

It will be evident that one of the biochemical processes of aging consists in a lowering of the biochemical activities, if one takes in consideration the facts which have accumulated up to the present time, namely: The important role of PABA which is liberated in the organism via hydrolysis in the methylation process [16, 17]; our data on the intensification of the methylation process in the spleen of old rats by novocain; the investigations of Gaffney [26] on the lowering with age of the Vitamin B_{12} content, which is one of the activators in the methylation process; the observations of Mothes [27] who linked the aging process in plants to a lowering in methylation; and our data on the exchange transformation of cysteine, cystine, methionine, and aspartic and glutamic acids in old animals.

One may assume that cysteine plays an important role in the process of tissue regeneration, by taking in consideration the fact that in old animals a prolonged administration of cysteine leads to a sharp rise in blood serum methionine. This is supported by the results of serine administration to old rats, which causes a lowering in cystine and a rise in cysteine contents [28], as well as the increase in cysteine in the liver and kidneys by PABA which is literated in the organism via hydrolysis.

In this connection one should also mention data from the field of radiobiology, according to which cysteine exerts a protective effect in radiation injuries, counteracting, in particular, the very intensification of the aging process caused by radiant energy [29].

To the investigations on animal organisms one should add the studies of Mothes [27] on plants, according to which the aging of plants is linked to a lowering in the number of sulfhydryl groups. If one adds to these experiments those data showing that the reduction of cystine to cysteine takes place simultaneously with the conversion of oxidized glutathione into its reduced form, as well as the investigations on the markedly increased oxidized glutathione content in old animals and its decrease to a level found in young animals under the effect of cysteine, it is therefore evident that the ratio of free cysteine/cystine and their contents in a bound state in the glutathione complex, as well as the ratio of cysteine/cystine and methionine contents are changing with age.

The oxidation-reduction mechanism, represented by the systems cysteine-cystine and reduced glutathione-oxidized glutathione acquires special interest if the aging process is considered as a result of prolonged effect of numerous toxic substances, among which cystine plays an important role (as evidenced in particular from its toxic effect upon the liver, as against the therapeutic effect of cysteine in liver dystrophy). It can be seen from the experimental data that one of the most important mechanisms of the process of aging is reflected in the balance between the sulfhydryl and the disulfide groups, which in turn are closely linked to the process of demethylation.

SUMMARY

The results of the investigations show that in the process of aging in the animal organism (rats, guinea pigs, rabbits, and dogs) there takes place a demethylation, evidenced by a lowering in methionine content, and accompanied by an accumulation of disulfide groups (cystine and oxidized glutathione).

The administration of substances containing sulfhydryl groups (cysteine) to old animals brings about a restoration of the biochemical equilibrium observed in young animals.

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ARGININE DECARBOXYLASE IN Salmonella typhimurium

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Translated from Biokhimiya Vol. 25, No. 1, pp. 68-72, January-February, 1960

Original article submitted May 26, 1959

Amino acid decarboxylases are widely distributed in microorganisms of the intestinal group. The dysentary group, intestinal bacilli, salmonellae and some other microbes have five different amino acid decarboxylases: glutamic acid, ornithine, arginine, histidine, and lysine. For microorganisms of the dysentary group and intestinal bacilli glutamic acid decarboxylase is a constant enzyme, while the other amino acid decarboxylases evidently are adaptive enzymes, of which ten are absent in different strains of the microbes [1]. Salmonella, on the contrary, are characterized by the complete absence of glutamic acid decarboxylase. According to our data [2], most characteristic for this groups of microorganisms is the presence of arginine decarboxylase.

In the present work we give data on the study of arginine decarboxylase in Salmonella typhimurium obtained from cell-free preparations, and the possibility of its purification.

METHODS

As a starting strain for the study of arginine decarboxylase activity we used the strain Salmonella typhimurium No. 37 grown on kitten liver in the Rostov Institute of Epidemiology, Microbiology, and Hygiene. This strain had the ability to decarboxylate only arginine. Other amino acids (histidine, lysine, and glutamic acid) were not decarboxylated by this strain.

The culture was grown at 37° for 18-20 hours on a meat-peptone agar with the addition of 2% glucose. The glucose was added because under these conditions we observed in the microbes an increased enzyme activity compared to microbes grown on a medium without glucose.

Determination of arginine decarboxylase was carried out by a manometric method in the Warburg apparatus. We used a preparation which consisted of whole cells of Salmonella typhimurium and a cell-free preparation. To obtain the preparation of the first type the cultured cells were washed from the solid nutritive medium with physiological saline, washed twice more, and collected by centrifuging. The experiments with whole cells were carried out in an atmosphere of nitrogen.

The cell-free preparations were obtained by extracting the cells with some buffer which caused considerable destruction of the bacterial cell. This destruction of the cell was attained in the homogenizer. For this purpose a thick bacterial mass, washed free of nutritive medium by centrifuging, was placed on the friction surface of the homogenizer and then ground with the addition of glass powder. The rotation of the internal part of the homogenizer was produced with a rotating device (in our case an electric drill). Small portions of glass powder were added during the grinding. The homogenizer was placed in a cooling mixture to prevent overheating. Destruction of the cells was carried out for 15-20 min; then the homogenizer was filled with buffer solution and the mixture was agitated for another 5-10 min. We used a citrate-phosphate buffer, pH 7.6, as the solvent. The use of a medium with this pH assured getting the most active extract. After destruction of the cells, the mixture stood 2 hours at room temperature with periodic shaking. Then the mixture was centrifuged for 30 min at 6000 rpm. The clear supernatant liquid was treated with six times its volume of cooled acetone. The resulting precipitate was collected and dried on a Buchner funnel by washing with suction, at first with acetone, then with a mixture of acetone and ethyl ether, and finally with ether. We obtained a white, powdery material. It is necessary to add that preparation of the acetone powder from whole cells followed by extraction of the enzyme from the powder with the same buffer gives an extract with lower activity.

Nitrogen in the preparation was determined by the Kjeldahl method, and phosphorus according to Fiske-Subbarow. The optical density of the solution was measured with an SF-4 spectrophotometer.

RESULTS

The effect of pH of the medium on the activity of the whole cell and cell-free preparations of arginine decarboxylase is shown in Figs. 1, A, B.

Whole bacterial cells decarboxylate arginine most actively in a rather acid medium at pH 3.6, while at pH 6.0 decarboxylation hardly occurs. We must consider, of course, that with increase in pH the amount of CO_2 going into solution is sharply increased and obviously the results of the determinations at pH 6.0, 6.5, and 7.0 will be low.

The cell-free preparation of arginine decarboxylase from Salmonella typhimurium also showed optimum activity at pH 3.6. Hence the low value for the optimum pH for decarboxylation of arginine is a property of this enzyme independent of the permeability of the cell wall for the substrate at low pH values when the activity is determined in whole cells. In the cell-free extract the enzymatic

activity calculated on 1 mg of dry weight of substance increased 2.85 times.

For further purification of the enzyme to obtain a more active preparation we took advantage of the possibility of fractionating the protein with ethyl alcohol. This method has an advantage over other methods of fractionation in the possibility of easy removal of this solvent and of getting dry preparations.

However, it is important not to inactivate the enzyme by contact with the alcohol.

The use of ethyl alcohol for fractional precipitation of proteins is based on the fact that alcohol decreases the dielectric constant of aqueous protein solutions. Since the solubility of a protein depends on the dielectric constant of the solvent, this method can be used for stepwise decrease in the solubility of the protein, and hence for its fractionation [3]. This method was previously purely empirical and the results of its use were more or less accidental. The method has been much improved by Cohn, Edsall, Oncley, and others [4].

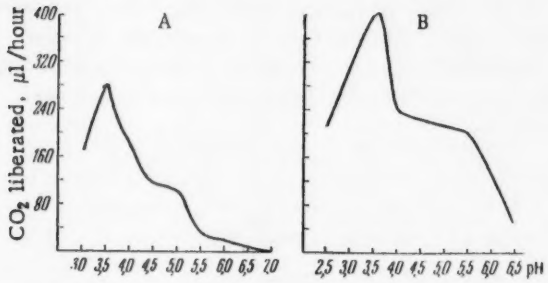

Fig. 1. Effect of pH of the medium on activity of arginine decarboxylase of whole bacterial cells (A) and cell-free extracts (B). In the Warburg vessel are 2 ml of citrate-phosphate buffer; 2 ml 1/30 M arginine and 1 ml of cell sample in water (A) or 1 ml of sample containing 1 mg of acetone powder (B).

For the least possible denaturation and inactivation of the enzyme during fractionation, precipitation was carried out at 2°. The alcohol was added slowly with constant stirring of the solution. Fractions were collected by centrifuging immediately after precipitation. After centrifuging, the supernatant liquid was poured off and the dense precipitate, right in the centrifuge tube, was dried in a vacuum desiccator. Thus there was the minimum time of contact of the enzyme with the precipitant, alcohol. For precipitation we used 96% alcohol which had first been redistilled through a small rectifying column.

For fractionation the preparation of acetone powder was dissolved in a buffer with pH 4.0. This pH value was chosen because the optimum action of this enzyme lies at pH 3.6. The optimum pH of enzyme activity often (but not always) is close to its isoelectric point. The lowest stability of the protein at the isoelectric point or close to it should, in our opinion, lead to the most rapid precipitation of the enzyme from the solution when ethyl alcohol is added to it. We carried out these fractions at different concentrations of the preparation in the solution. In each case we obtained a different number of fractions. This could be quite large (15-20 fractions).

Fractionation of Solutions of Acetone Preparation by Ethyl Alcohol. (For fractionation we took 20 ml of a 1% solution of the preparation; fractionation was carried out at 2°)

Fraction No.	Amt. of alcohol added, ml	Yield of preparation, mg	Enzymatic activity, µl CO_2 after 1 hour per mg dry substance
1	5	9,2	200
2	1,5	5,7	200
3	1,5	11,3	360
4	1,5	12,5	800
5	1,5	10,0	600
6	1,5	13,5	420
7	1,5	18,3	200
8	1,5	20,8	120
9–16	1,5×8	79,0	Activity absent

The best results were obtained under the following experimental conditions. We prepared a 1% sample of the acetone preparation. The insoluble part was separated by centrifuging. To 20 ml of the solution we added at first 5 ml of 96% alcohol. The precipitate was designated as fraction 1. Each further successive fraction was collected after adding 1.5 ml of alcohol. As a result of this procedure we collected 16 fractions. To establish the presence of enzymatic activity in each fraction we used the method which we had worked out earlier for detecting decarboxylation of amino acids by identifying the corresponding amine by paper electrophoresis [5]. This method in the course of two hours showed which fractions had activity. Then the activity of the fraction was further studied in the Warburg apparatus for quantitative estimation of the activity.

The results of the experiments on fractionation of solutions of the acetone preparation by ethyl alcohol and the determination of enzymatic activity in the resulting fractions are given in the table.

Under the above experimental conditions fractions 4 is the most active. In other experiments fractions 4 or 5 were active. Fractionation of the solution of the acetone preparation by ethyl alcohol permits us to get a preparation with an activity about 20 times greater than the activity of the acetone powder, calculated on the dry weight.

Beginning with fraction 6 we observed a rapid decrease in enzymatic activity, and as a rule, fractions 10-12 had already lost their activity. We explained this partly by the removal of enzyme in the first fractions, and also by the loss in enzymatic activity of the remaining substance due to the high concentration of alcohol, since in separate experiments the addition to the enzyme solution of an equal volume of 96% alcohol (to a final concentration of 47.7%) suppressed the enzymatic activity. The most

active fractions separated from the preparation were combined and submitted to chemical analysis. Their N content was 13%, P 2.5%.

As Fig. 2 shows, the maximum optical density of the solution of enzymes lay at 260 mμ (Fig. 2). Such an absorption maximum is characteristic for nucleic acids [6]. Since our preparation contained a considerable amount of phosphorus (2.5%), the question arises whether the enzyme activity is connected with nucleic acids. We therefore studied the action of crystalline deoxyribonuclease on the enzyme preparation. The action of deoxyribonuclease on the enzyme was estimated by the change in viscosity of the original solution. However, no change in viscosity was found during the experiment and arginine decarboxylase activity did not disappear. Hence the enzyme solution did not contain high molecular weight nucleic acids, but rather products of the type of the nucleotides which determine the absorption in the ultraviolet at 260 mμ.

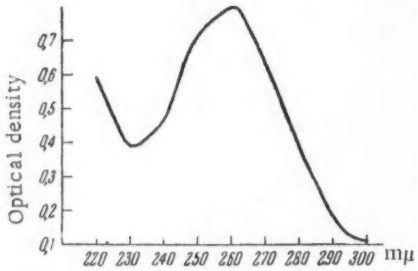

Fig. 2. Ultraviolet absorption of arginine decarboxylase solutions.

We then tried to separate the protein portion of the preparation in solution from the nonprotein by treating the enzyme solution with chloroform. Actually, we succeeded in obtaining a very small amount of protein from the solution, but neither the protein nor the protein-free part remaining had enzymatic activity. Enzymatic activity evidently also depended on this small amount of protein.

Thus, as a result of fractionation by ethyl alcohol of the solution of acetone powder isolated from the bacterial extract, we obtained an enzyme preparation with a sufficiently high activity and which also contained fragments of nucleic acids as a contaminant.

Since we had an active preparation of arginine decarboxylase at our disposal, we decided to use it for the preparation of the product of decarboxylation of arginine, agmatine.

For this purpose, the arginine solution in the citrate-phosphate buffer, pH 4.0 was treated with the enzyme preparation and the mixture was left in the thermostat for 5 hours. To prevent entry of microorganisms, we covered the mixture of substrate and enzyme in the flask with a thin layer of toluene. After the time interval mentioned, the toluene was removed and the mixture boiled to remove the enzyme preparation; it was filtered and the filtrate served for the isolation of agmatine. In this case it was necessary to remove agmatine from the salts of the buffer mixture and the arginine which was not involved in the process of decarboxylation (in our case, we used DL-arginine).

Isolation of agmatine was carried out on ion exchange resin KU-2. KU-2 resin is a monofunctional cationite with sulfo groups [7]. The resin was first ground to 0.1 mm mesh. The ground resin was soaked in 5 N HCl for 18 hours and then washed 6 times with the same acid solution by decantation, and then was washed with water to a neutral reaction. Then the resin was "trained." This included alternate treatment of the resin with acid and alkali. This procedure was carried out in the following order: treatment with 1 N HCl – water – 0.5 N NaOH – water – 1 N HCl – water to a neutral reaction to methyl orange. This cycle of treatment was repeated 15 times. Then the resin was given one cycle of treatment: 1 N HCl – water – 0.1 N NaOH – water – 1 N HCl – water to a negative reaction to methyl orange. The resin treated in this way before use was put in a 20% acetic acid solution for 18 hours. Immediately before the experiment it was washed with 20% acetic acid 5 or 6 times, packed in a column and again washed with 300 ml of 20% acetic acid solution and finally with water to a neutral reaction.

We passed through the column (0.8 × 40 cm) filled with "trained" resin a mixture of arginine and agmatine in a citrate-phosphate buffer, pH 4.6. After passing the solution, the column was washed with 100 ml of distilled water. The presence of arginine and agmatine in the eluate was controlled by electrophoretic separation. After the column had been washed with water, no amino acid or amine was found in the eluate. Then the column was washed with 100 ml of 10% NH_3 solution. The eluate in this case showed no ninhydrin positive substances. Washing with ammonia was continued until salts disappeared from the eluate. The column was then washed with 1 N HCl. This wash liquid also contained a large quantity of salts and the amino acid arginine. Agmatine was not found. Washing with HCl was ended when the wash liquid from the column contained no more salts or arginine. This required more than 5000 ml of HCl.

Finally, the column was washed with 3 N HCl. Only agmatine was found in the eluate. However, the washings of wash liquid were then colored greenish. To remove the substance which gave the green color, we mixed the concentrated eluate with activated charcoal, boiled it and filtered hot through a dense paper filter. When the filtrate was evaporated in a vacuum, white crystals of agmatine hydrochloride formed.

Thus, the above procedure permits separation of a mixture of arginine and agmatine and the isolation of agmatine as a preparation.

SUMMARY

We have studied the arginine decarboxylase of Salmonella typhimurium. The optimum pH for the

decarboxylase activity in whole cells and cell-free preparations lies in the acid medium at pH 3.6.

The cell-free preparation obtained by extraction of the cells destroyed in a citrate-phosphate buffer at pH 7.6 was submitted to fractionation with ethyl alcohol. As a result we obtained fractions with an activity 20 times greater than that of the original extract. The most active fraction was not free from nucleotides, since the ultraviolet absorption maximum for its solution was at 260 mμ.

The resulting enzyme preparation was used for preparation of agmatine. Agmatine was isolated from a mixture with arginine in a salt buffer using cationite KU-2.

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THE EFFECT OF IRRADIATION ON SOME STEPS IN THE OXIDATIVE TRANSFORMATION OF CARBOHYDRATES IN RAT LIVERS*

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Translated from Biokhimiya Vol. 25, No. 1, pp. 73-79, January-February, 1960

Original article submitted June 2, 1959

As a result of a large number of investigations have shown, the most radiosensitive tissues in the animal organism are the bone marrow, lymph nodes, spleen, thyroid gland, and liver. In these organs, along with some others, there is a very high degree of activity of enzymes for the direct oxidation of glucose. The destruction of carbohydrate metabolism by irradiation has been observed in almost all studies of organs and tissues, but it is shown especially clearly in the liver. In this connection we decided to follow the effect of radiation on different stages of the oxidative pathway for utilization of hexoses in the liver.

Our experimental results on this question make up contents of this paper.

METHODS

We used female white rats, weight 130-185 g, in the experiments. The animals were totally irradiated with γ-rays of Co^{60}. The distance from the source of radiation to the middle of the body of the animal was 35 cm; power of the dose 17 r/min. Twenty-four hours after the irradiation, during which time both the controls (unirradiated) and the experimental rats received only water, the animals were sacrificed by decapitation, and the liver was taken for study by making the enzyme preparations from it [1]. The activity of the G-6-P and 6-PG1 dehydrogenases was measured by the rate of reduction of TPN as determined by change in optical density at 340 mμ (on an SF-4 spectrophotometer, thickness of cuvette 1 cm). The composition of the sample in these determinations was: 0.5 ml 0.1 M $MgCl_2$; 0.5 ml 0.25 M glycyl glycine buffer (at pH 7.6 for G-6-P dehydrogenase and pH 9.0 for 6-PG1 dehydrogenase); 0.2 mg TPN and 0.1 ml 0.05 M substrate to a final volume of 2.5 ml. Reaction was initiated by adding 0.1 ml (in some experiments, 0.05 ml) of enzyme preparation. The dehydrogenase activity was determined spectrophotometrically, measuring the rate of reduction of TPN at 340 mμ at room temperature (20-22°). G-6-P dehydrogenase activity was measured as the difference between the activity measured at pH 7.6 in the presence of 0.1 ml of solution containing G-6-P and 6-PG1 (both at a concentration of 0.05 M) and the activity in the presence of 0.1 ml of 0.05 M 6-PG1 solution, also measured at pH 7.6. Spectrophotometer readings were made each minute for 5 min. In each case we used a sample without adding TPN and the results found in this sample were subtracted from the values obtained for the samples with added TPN.

The process of splitting of R-5-P and the phosphatase activity of liver extracts with respect to F-1,6-P were estimated after incubation in a thermostat at 30°. The intensity of the first process was judged by the decrease in ribose; in the second case we determined the increase in inorganic phosphate.

TPN was obtained by the method of Warburg [2] in the modification of Dickens and Glock [3]; purification reached the third stage (according to Warburg); HMP was obtained by the method of Dubois and Potter [4]. The preparation did not contain inorganic phosphate and contaminants amounted to 18%; the HMP contained 98.5% of the total phosphorus. The preparation of 6-PG1, obtained by oxidizing HMP with bromine [5] contained practically no inorganic phosphorus and gave a negative reaction for fructose. F-1,6-P was obtained by fermenting cane sugar in the presence of toluene [6]; the purity of the preparation was 77% with a slight content of inorganic phosphorus. To obtain R-5-P we carried out acid hydrolysis of ATP [7]. The resulting preparation contained 3% inorganic phosphorus (with repeat to the total phosphorus) and did not contain nitrogen; the purity of the preparation was 96%. In the experiments we used solutions of the potassium salts of the above-mentioned substrates, neutralized to pH 7.0. Glycyl glycine was obtained from glycocol [6]; the content of impurities in it was 1% (according to the results of determination of amino nitrogen before and after acid hydrolysis).

For the determination of phosphatase activity and splitting of R-5-P during incubation, we took samples from the reaction mixture with a volume of 0.5 ml and precipitated the protein from the solution with $HClO_4$ (final con-

* We have used in this paper the abbreviations: G-6-P – glucose-6-phosphate; 6-PG1 – 6-phosphogluconate; TPN – triphosphopyridine nucleotide; R-5-P – ribose-5-phosphate; F-1,6-P – fructose-1,6-diphosphate; HMP – hexose monophosphate; Gl-3-P – glyceraldehyde-3-phosphate; F-6-P – fructose-6-phosphate; S-7-P – sedoheptulose-7-phosphate.

centration 0.27 N). After centrifuging, we determined inorganic phosphorus by the reaction with molybdenum reagent [6]; R-5-P and S-7-P were determined by the reaction with orcinol [8], using the formula suggested by Horecker et al., [9], and hexoses were determined by the first method of Dische et al. [10] with some modifications [11].

RESULTS AND DISCUSSION

As a result of irradiating rats with doses of 600 r the activity of 6-PG1 and G-6-P dehydrogenases in the liver calculated per g of wet weight decreased in one experiment and increased in others. The decrease in activity of the dehydrogenases, and even more, the increases exceeded the limits of individual variation or the experimental errors of the determination and depended on the effect of the irradiation. The change in enzyme activity in opposite directions under the influence of radiation is only seeming. In fact, after irradiation there was a considerable increase in wet weight of the liver (in some cases more than 40%), and the observed difference in weight could not be explained only by hydration of the organ, after irradiation, since the decrease in dry weight per g of tissue did not occur to such an extent as to compensate for this difference in weight. It follows from all this that in this case the dehydrogenase activity must be calculated, not per g of wet or dry weight of liver, but on the weight of the organ. With such a calculation, the results are different: In experiments which show inhibition of the activity of the enzyme being studied there is now almost no difference between the control and the experimental animals, and where there is dehydrogenase activity, the effect of irradiation is strengthened (Fig. 1).

The degree of activation of the dehydrogenases varied in rather wide limits, but did not depend on dose of irradiation. However, with increasing dose of radiation there was an increase in the percentage of animals which reacted with an increased dehydrogenase activity; at 1200 r this number was 100%. A direct relation between increased activity of dehydrogenase in the liver and change in its weight was not observed.

We should remark that not only in radiation experiments, but in all cases where there is a change in weight of the organ, the principle of calculating the enzyme activity on the weight of the organ is evidently more accurate (at least for the liver) than per gram of dry or moist weight. This is also fully justified for normal animals, since the size of individual variation in enzyme activity (in the liver) calculated on the weight of the organ is much less than when calculated per gram of moist or dry weight. When the difference in weight of the organ is considerable, as occurs, for example, in our experiments with irradiated animals, the principle of calculating on the weight of the organ can be decisive for a true interpretation of the data which we obtain.

In the study of the effect of irradiation on the process of splitting of R-5-P in liver tissue, the rats were irradiated

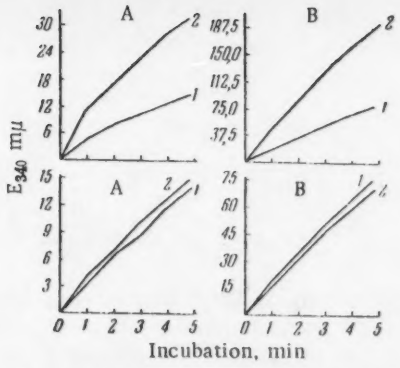


Fig. 1. Activity of G-6-P and 6-PG1 dehydrogenases after irradiation. Optical density given by calculation over to weight of organ. 1) Control rats; 2) irradiated with a dose of 600 r. A) Activity of G-6-P dehydrogenase; B) activity of 6-PG1 dehydrogenase.

with a dose of 600 r. As Fig. 2 shows, the decrease in ribose in the experimental and control animals was the same and occurred very intensively in the first 60 min after beginning incubation; then it stopped. Stabilization of the ribose content corresponded to the moment when, in the incubated sample, there remained about 25% of unreacted original substrate independent of its original concentration. Utilization of ribose was accompanied by the formation of heptulose† which in the first 60 min occurred very intensively, just as was the case with the fall in ribose, and occurred with the same rate in both experimental and control samples (Fig. 2). (In these graphs we give the data for the amount of ribose and heptulose in the incubated samples after 0, 60, 90, 120, 150, and 180 min. However, in some experiments we also carried out determinations after 10, 20, and 30 min. The resulting curves did not differ from those which are given in Fig. 2. Beginning with 60 min, the amount of heptulose decreased, more strongly in the control samples than in the experimental.

The slowing of the fall, or stabilization of the content of heptulose in the later stages of incubation can be caused by inhibition of the transaldolase activity or by lack of G1-3-P which serves as an acceptor for the dihydroxyacetone group in the transaldolase reaction (scheme).

The splitting of R-5-P in our experiments occurred at the same rate in experiments and controls, and thus the

† The method which we used for detecting heptulose determined free heptulose and also its phosphorus derivatives. However, since in the transketolase reaction from R-5-P as the starting substrate only S-7-P was formed, there was no basis for assuming that in our experiments any other heptulose compound was formed. Therefore all the heptulose which was determined in our experiments was calculated as S-7-P.

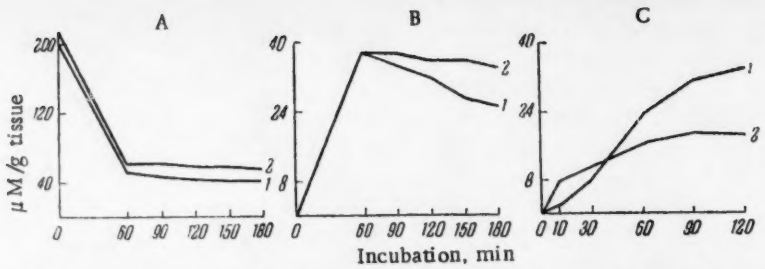
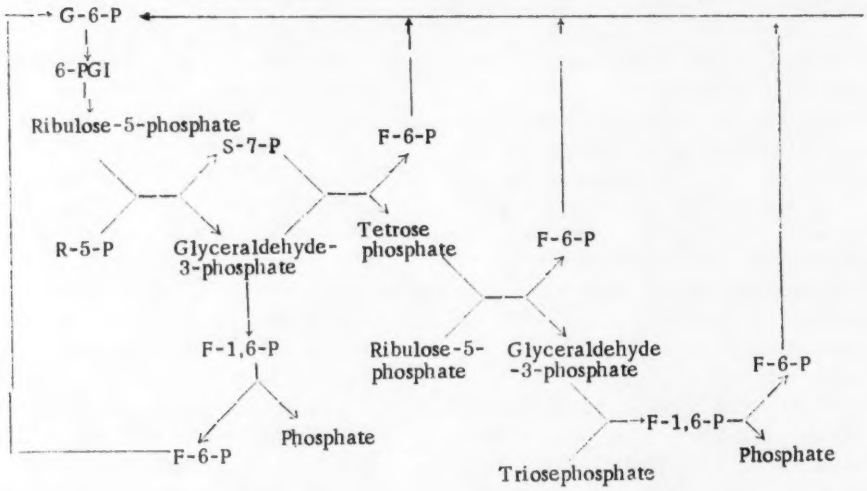


Fig. 2. Effect of irradiation on utilization of R-5-P (A) and formation of heptulose (B) and hexoses (C) in rat liver extracts. Composition of samples: 0.75 ml 0.25 M glycyl glycine buffer, pH 7.6; 0.3 ml enzyme extract of liver and 6.6 μM R-5-P. Final volume 3.75 ml. Time of counting incubation started 1 min after putting the tube in the thermostat at 30°. In this and all following experiments we took samples without a substrate, the data of which were subtracted from the values obtained in samples with added substrate. 1) Unirradiated animals; 2) irradiated at a dose of 600 r.

Scheme of Direct Oxidative Pathway for Carbohydrate Breakdown



amount of G1-3-P formed should be the same. However, we must consider that along with the formation of G1-3-P there occurs also its continuous condensation into F-1,6-P and splitting of the latter into organic phosphate and F-6-P. Therefore, the increase in activity of F-1,6-P phosphatase would equally strongly decrease the amount of G1-3-P in the incubated samples until it would disappear at the last stages of incubation, when its formation would cease in connection with the absence of decrease of ribose. The disappearance from the system of the dihydroxyacetone acceptor would also lead to slowing or stoppage of the transaldolase reaction. Thus, the rate of splitting of heptulose will depend on the ratio of the rates of two processes, the transaldolase reaction and the splitting of phosphoric acid radicals from F-1,6-P. The greater the rate of the first process and the lower that of the second, the more energetically will the decrease of heptulose occur, and vice versa.

Irradiation with a dose of 600 r caused a distinct increase in activity of fructose diphosphatase (Fig. 3), and the resulting difference in activity between control and experimental samples shows a decreased characteristic change in enzyme activity, since the rate of the process in this case depends in only slight degree on the activity of the enzyme, in view of its great concentration in the system. It follows from all this that the slowing in the fall of heptulose in the experimental samples as found in our investigation is explained by the lessened content of G1-3-P which is an acceptor in the transaldolase reaction.

This conclusion agrees with the curves of formation of hexose‡ in the process of splitting R-5-P (Fig. 2). In the early stages of incubation there is intensive formation of hexose in the experimental samples, then it is somewhat lower, and after about 60 min it practically stops. In the controls, the formation of hexose at first goes to a slight

‡Under this term we include the free hexoses, glucose and fructose, and their phosphate derivatives, that is, G-6-P, F-1,6-P, and F-6-P; when the cysteine method is used, all these compounds are determined together.

extent, then increases, and does not stop during the whole period of incubation. This difference is well explained by the higher activity of F-1,6-P phosphatase in the experimental samples. Actually, if at the beginning of incubation there is intensive splitting of R-5-P and, correspondingly, formation of a considerable amount of Gl-3-P, then in the presence of highly active F-1,6-P phosphatase, it changes into F-6-P which destroys the equilibrium between F-1,6-P and the phosphotrioses, and normal occurrence of the transaldolase reaction becomes impossible. This should lead to stopping or decrease in accumulation of hexose in the latter stages of incubation because of splitting of heptulose (scheme), which actually occurs. The hexose formed at the beginning of incubation in the experimental samples could be removed because of F-6-P formed from triose phosphate by means of F-1,6-P. Naturally in the control samples, where phosphatase is less active, the amount of F-6-P formed in this way should be less. Since at the beginning of incubation the initial amount of substrate, S-7-P, is zero, the transaldolase reaction will proceed slowly so that in this way there should be formed only a slight amount of F-6-P. Hence, also, the total amount of hexose should be less than in the experimental samples. With further splitting of R-5-P and formation of heptulose, the splitting of the latter should occur more intensively and should be accompanied by more intensive accumulation of hexose. The decrease in heptulose will not stop, and hence the increase in hexose should also occur to the end of incubation (Fig. 2). The difference in amount of hexose formed to the end of incubation can be explained by the fact that in the transaldolase reaction the prevailing method of hexose formation in the control samples is from two molecules of R-5-P to form one molecule of hexose, and in the experimental samples the hexose is formed through F-1,6-P, 0.5 molecule, that is, half as much.

We noted above that the fall in ribose in the liver extracts of irradiated and nonirradiated animals occurred at the same rate. However, on the basis of these experiments we cannot judge the actual ratio of activities of enzyme which splits R-5-P since its concentration in the system can be considerable. With a threefold decrease in enzyme extract, the disappearance of ribose does not occur so intensively as before, but a greater intensity of splitting of R-5-P in the experimental samples is clearly shown only by calculating the data on weight of organ. It is true that analogous relations, less clearly shown, are found in the process of formation of heptulose. More convincing data were obtained by decreasing the amount of enzyme six-fold, when obvious activation of the process of splitting R-5-P after irradiation was found even with calculations on 1 g of moist weight (Fig. 4).

The assumption that increased formation of hexose in the experimental samples in the early stages of incubation occurs as a result of intensification of the phosphatase reaction finds confirmation in a study of the process of formation of hexose in experiments with decreased amount of enzyme extract.

When the enzyme concentration is decreased threefold, the formation of hexose in the control after 60 min of incubation is practically not observed, and only later does its amount begin to increase markedly. This inhibition of hexose production is explained by the fact that splitting of ribose in this case (Fig. 4) occurs more slowly than when higher concentrations of enzyme extract are used. In the experimental samples, where the formation of hexose occurs basically through F-1,6-P, it is found even at the beginning of incubation. Since the splitting of ribose phosphate does not stop, and hence the formation of triose phosphate also does not stop, the amount of hexose with continually increase in distinction from the experiments where splitting of ribose stops, and hence also formation of hexose, even after 60 min.

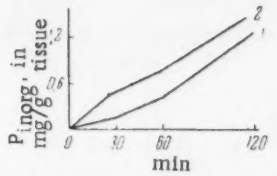


Fig. 3. Effect of irradiation on fructose diphosphatase activity of the liver. Composition of sample: 0.5 ml 0.25 M glycyl glycine buffer, pH 7.6; 10 μM F-1,6-P and 0.2 ml of enzyme preparation to a final volume of 2.5 ml. Incubation temperature 30°. 1) Unirradiated animals; 2) irradiated with a dose of 600 r.

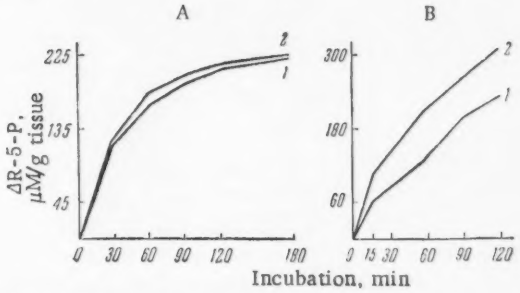


Fig. 4. Effect of irradiation on decrease in R-5-P. Designations of curves and course of experiments the same as in Fig. 2, except that in this case the amount of enzyme extract in the sample in graph A is 3 times less, and in graph B, 6 times.

With further decrease in concentration of the enzyme preparation the interrelation is shown still more clearly (Fig. 5). This is shown by the fact that in the control samples formation of hexose in general does not occur, a fact which agrees well with the slower splitting of R-5-P

than in the previous experiments (calculated on separate samples), and hence the slower formation of S-7-P. The formation of hexose in the experimental samples occurs as before, intensively during the whole incubation, but is completely stopped when strong acids are added to the sample (to bind Gl-3-P). This fact shows that hexose formation in the experimental samples actually goes through F-1,6-P.

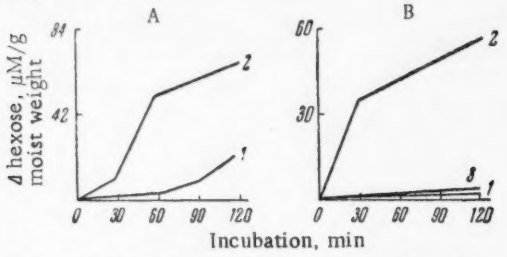


Fig. 5. Effect of irradiation on hexose formation. Experimental conditions the same as in Fig. 2; only the amount of enzyme extract is correspondingly decreased threefold (Graph A) and sixfold (graph B). 1) Unirradiated animals; 2) irradiated with a dose of 600 r; 3) irradiated with 600 r with addition in the latter case of KCN (final concentration 0.01 M) to the incubated sample.

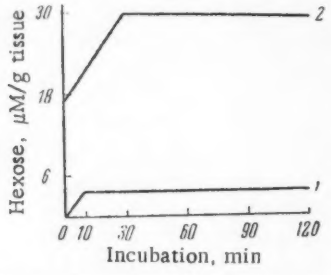


Fig. 6. Formation of hexose when the liver extract is incubated without addition of substrate. Designation of curves and conditions of experiment the same as in Fig. 2.

Thus, the picture which was found at higher concentrations of enzyme in the early stages of incubation comes at a later period when the enzyme concentration is decreased. This regularity in the control samples assures a slowing in rate of splitting of R-5-P, a decrease in the amount of S-7-P formed, and a slight rate of its further transformation by the transaldolase pathway. At the same time, in the experimental samples the condensation of phosphotriose is hastened because of the shift of the equilibrium to the side of F-1, 6-P so that when the enzyme extract is diluted the difference is more sharply shown in the action of fructose diphosphatase which is more active in the experimental samples.

In all the experiments in which R-5-P was used as the substrate, control samples were also run without adding substrate. It was shown that ribose and heptulose were absent in it both before and after incubation, while hexose was observed in the liver of nonirradiated animals only in small amounts and not in all experiments, while in the irradiated animals it was present in considerable amounts and in all the animals studied. As a rule, the quantity of hexose did not change substantially during incubation, but in a number of experiments we noted an increase in its content, but only in the early stages of incubation (Fig. 6). The fact that under the influence of irradiation the rate of hydrolytic splitting of glycogen in rat livers [12] is increased leads us to believe that in our experiments the high content of hexose in the liver of irradiated rats assures intensification of this process.

SUMMARY

Total irradiation of rats with doses of 300, 600, 900 and 1200 r causes a decrease in dry weight of 1 g of liver and an increase in moist and dry weight of the organ as a whole. Quantitative dependence on dose was not observed.

The activity of G-6-P and 6-PG1 dehydrogenases in the liver at doses of 600 r increased in one case, and did not change in others. When the dose was increased irradiation caused an increase in the number of animals which showed a change in activity of the enzyme, and at 1200 r the dehydrogenase activity was increased in all the animals.

In the liver after irradiation with a dose of 600 r, the splitting of R-5-P and formation of S-7-P occurred faster; increase in fructose diphosphatase activity occurred; the hexose content in the tissues increased and the process of its formation was considerably changed, both qualitatively and quantitatively (using R-5-P as substrate).

In conclusion we express our thanks to Prof. S. E. Severin for his suggestions and guidance in carrying out the work.

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THE DETERMINATION OF "FREE" VITAMIN B_{12} IN BLOOD SERUM WITH B. Coli

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Translated from Biokhimiya Vol. 25, No. 1, pp. 80-85, January-February, 1960

Original article submitted June 2, 1959

The question of the relation of "free" vitamin B_{12} to that bound to proteins or polypeptides in the tissues and biological fluids and the importance of each of these forms of the vitamin in metabolic processes has been little studied up to now. A study of this question can cast light on the mechanism of assimilation of the vitamin under different conditions of its introduction into the organism and transfer within it, and can also be of interest in explaining the function of the vitamin in normal and pathological states, especially its role in biosynthesis and metabolism of proteins [1, 2]. However, study of the significance and function of "free" cyanocobalamine at the present time is hindered by the difficulty of its determination along with the bound form of the vitamin, which usually predominates in tissues and biological fluids.

In the present work we give a new method for determining "free" vitamin B_{12} in blood serum with the help of B. coli 113-3 and describe the results of its determination in the serum of healthy individuals and of some hematological patients.

METHODS

As is known, most of the vitamin B_{12} in blood serum is bound with proteins, especially (73-93%) with α-globulines [3, 4], and only a small part is found in the "free" form. Our understanding of "free" vitamin B_{12} at the present time is very complex; by this term we usually mean that portion of the vitamin which can be determined microbiologically without preliminary splitting of the vitamin to protein bound at 100° or higher temperatures, that is, that portion of the vitamin which is bound weakly or not at all to protein. The most suitable method for the determination of the "free" vitamin in blood serum and other biological fluids is the method of Ross, based on determination of the vitamin in serum heated only to 56° for 30 min [5]. Such treatment, in the opinion of the author, does not liberate the vitamin from its bond with protein, but is necessary for further microbiological determination. The conditions for the determination of the "free" vitamin are shown by the experiments of Ross himself [6]: Heating dilute serum for 30 min at different temperatures, he obtained figures for vitamin activity which differed greatly from each other. Thus, the destruction of the vitamin-protein bond certainly depends to a great extent on the temperature used in the treatment of the serum, and it is not impossible that destruction of the vitamin-protein bond can also occur at 56° or below.

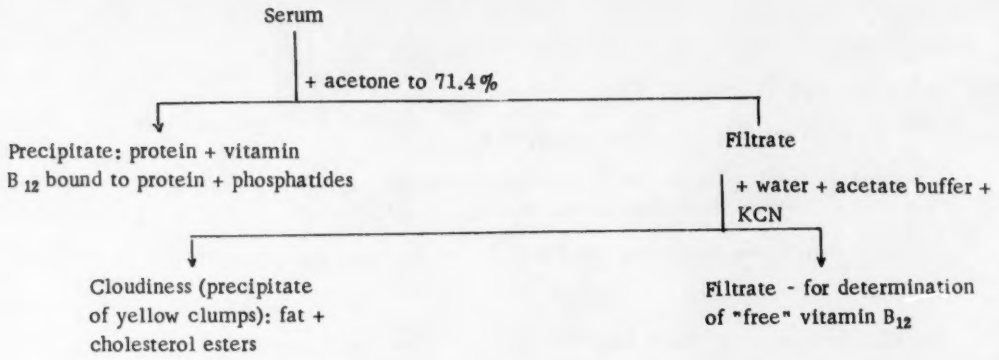
The Ross method requires use of a test microorganism of high sensitivity, such as Euglena gracilis; only in this case can the serum be diluted sufficiently so that protein will not affect the results of turbidimetric determination. A deficiency of the method is the necessity for sterile work at all stages in the preparation and treatment of the serum. Therefore to work out a method which does not require these conditions and permits sterilization of the experimental apparatus by autoclaving or boiling is of great interest, especially with determination of the vitamin with B. coli which is less sensitive to the vitamin.

The simplest process for separation of the "free" vitamin from the bound form seems to us to be precipitation of the protein by a substance which does not liberate the vitamin from its bond with the protein, does not react with the vitamin, and is easily removed, that is, does not affect the further course of the determination. The best precipitant for protein for these purposes is acetone. It is known that cyanocobalamine (and also hydroxycobalamine, vitamin B_{12B}) are fully soluble in 75% aqueous acetone, but are precipitated by 85-90% acetone [7-11]. Thus, our use for precipitation of protein of 71.4% acetone guarantees solubility of the vitamin and also assures full precipitation of the protein (protein is quantitatively precipitated even by 45% acetone; at lower acetone concentrations part of the protein appears in the filtrate, and at acetone concentrations below 40%, filtration is greatly slowed or does not occur at all). The aqueous acetone filtrate of the serum for further treatment is easily clouded by precipitation of water insoluble nonprotein substances of the serum, but this cloudiness can later be filtered out. We give the scheme of treatment of the serum below.

In order to test the method we determined the recovery of cobalamine in the treatment of a pure solution of known concentration by the above process. The recovery of the vitamin was 90-105%, that is, the variations did not exceed the experimental error of the method (±10-15%) [12].

We also tested the possibility of splitting the vitamin B_{12} bound to protein and its transfer to the "free" state under the influence of the acetone extract of serum.

Scheme of Treatment of Serum in Determination of Free Vitamin B_{12}


Serum
+ acetone to 71.4%
Precipitate: protein + vitamin B 12 bound to protein + phosphatides
Filtrate
+ water + acetate buffer + KCN
Cloudiness (precipitate of yellow clumps): fat + cholesterol esters
Filtrate - for determination of "free" vitamin B12

The blood serum of three of the 38 persons studied did not contain "free" vitamin B_{12}; the total content of vitamin in these sera was 430, 560, 715 $\mu\gamma$/ml. If there was even a partial splitting of vitamin bound to protein in the treatment with acetone, this would certainly be found as "free" vitamin. Also, when two of the three sera which did not contain "free" vitamin (the third was not studied) had a known amount of "free" and bound vitamin added to them, the only increase in total vitamin content, within the limits of error, was the amount of "free" vitamin. These facts show indirectly that our method of extracting the serum with acetone either does not affect, or affects to only a slight degree, the integrity of the vitamin-protein complex.

Course of the determination. To 4 ml of blood serum, taken under sterile conditions and kept in the cold, we added 10 ml of chemically pure acetone, shook, and filtered through a small paper filter, preferably with suction; the precipitate was washed with 2 ml of 70% aqueous acetone. To the filtrate we added 1 ml of acetate buffer with pH 4.63 ±0.02 (to assure the optimum pH of 5-6 for the thermal stability of the vitamin), 0.5 ml of KCN solution with a concentration of 0.1 g/liter, and 15 ml of twice distilled water. Then all the acetone was driven off from the slightly cloudy solution on the water bath (at 56-58° until the odor disappeared) and the solution was filtered through a small double filter into a sterile tube; the pH of the solution was brought to 6.8-7.0 with several drops of NaOH and the volume of the solution was adjusted to 20 ml with twice distilled water added through the filter. The tube was tightly stoppered with a sterile cotton gauze plug. The further determination was carried out by the method which we described earlier [12, 13]. Depending on the expected amount of "free" vitamin, we took for seeding 2-5 ml of filtrate (2 and 4; 2 and 5; 3 and 4; 2 and 5). In calculating low concentrations of the "free" vitamin, it was necessary to use not less than two parallel control experiments with the filtrate after destruction of the vitamin by alkali [13].

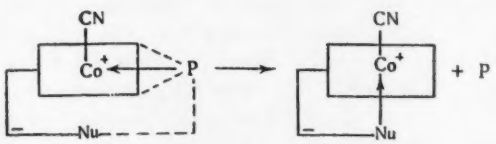
Our suggested method for determining "free" vitamin B_{12} by extracting it from serum with acetone with simultaneous precipitation of protein has, in our opinion, the following advantages over the method of Ross:

1) Complete separation of protein assures getting a perfectly clear filtrate and permits use of low dilution of the serum (1 : 5) which is very important for accurate results in calculating low concentrations of "free" vitamin in the serum;

2) Absence of protein and vitamin bound with it permits ordinary sterilization of the filtrate;

3) Removal of "free" cyanocobalamine by acetone probably has less effect on the integrity of the vitamin-protein complex than does thermal treatment.

The nature of the vitamin-protein bond is unknown at present. We can assume that it is multivalent with several bonds of different nature, and, therefore, of different stability. The complex of vitamin B_{12} with protein substances of the gastric juice, which contains the CN group of cyanocobalamine, cannot bind a second CN group [14, 15]. We cannot assume, then, that in this complex, and possibly also in others, there is a direct Co ← protein bond of the type Co←N where N is the nitrogen of a basic amino acid radical such as histidine [16, 17]. Splitting of the vitamin-protein complex can be represented in simplified form by the following scheme:



where P is protein, Nu the nucleotide part, and the rectangle is the porphyrin portion of the vitamin molecule. The strengths of the bonds for different proteins may be different: it is known that the vitamin-protein complex from liver homogenate which is active with respect to Euglena gracilis cannot be used for the bound vitamin of blood serum [18]. It is also important to consider that the vitamin activity of the "free" or bound vitamin can be different for different test organisms.

TABLE 1. Content of "Free" Vitamin B_{12} in Blood Serum of Healthy Individuals According to the Literature Data (vitamin B_{12} content in $\mu\gamma$/ml)

Author, year	"Free" vitamin		Total amount of vitamin		"Free" vitamin with respect to total amt. of vitamin, %	Method of determining "free" vitamin
	range	av.	range	av.		
Ross [6], 1950	20—350	185	350—750	490	37,7	Heating 30 min at 70°
Mollin, Ross [20], 1952	0—100	29	100—720	358	8,1	Heating 30 min at 56° [5]
Heinrich, Lachann [21], 1952	1—30	18	62—460	238	7,6	[5]
Goldeck, Weiss [22], 1953	25—40		280—350			[5]
Weiss, cited by [21], 1954	0—31	18	275—396	323	5,6	[5]
Lear, Harris, Castle, Fleming [23], 1954	27—104		292—856	532		[5]
Heinrich et al., [4], 1956					10,3	"Free" vitamin content calculated by amount of vitamin in electrophoretic fraction of γ + β-globulins
Raccuglia, Sacks [24], 1957	0—104	25	144—1080	387	6,4	[5]
Mai, Yavorkovskii [12], 1958	0—110	48	173—615	410	11,7	Removal with acetone and simultaneous precipitation of protein

TABLE 2. Content of "Free" Vitamin B_{12} in Blood Serum of Patients with Chronic Myelosis, in $\mu\gamma$/ml

Patient	"Free" vitamin B_{12}	Total vitamin B_{12}	Amount of "free" vitamin with respect to total, %
P	420	19800	2.1
N	129	14230	0.9
D	211	5177	4.1
Zh	300	1735	17.3
I	206	1538	13.4

The above mentioned uncertainty in determining "free" vitamin B_{12} can be removed only under conditions of purely physical separation of both forms of the vitamin: removal with organic solvents with simultaneous precipitation of the protein, dialysis, or (poorer), electrophoresis [4]. The first of these methods is obviously the simplest. A chromatographic method of separating "free" vitamin B_{12} has been described by Heinrich [19].

RESULTS AND DISCUSSION

"Free" vitamin B_{12} was determined by the above method in blood serum of 28 healthy individuals (primary donors) and then hematological patients (five with chronic myelosis, four with pernicious anemia in the stage of remission, and one with hemolytic anemia).

The amount of "free" vitamin in the serum of healthy individuals varies in rather wide limits, from 0 to 110 $\mu\gamma$/ml, with a variation in the total amount of vitamin of 173-617 $\mu\gamma$/ml; the percent of "free" vitamin in healthy serum averages 11.7% of the total amount of vitamin and varies from 0-45%. The results of the determination of "free" vitamin B_{12} in healthy blood serum obtained by various authors are given in Table 1.

Repeated determinations of "free" vitamin B_{12} in the serum of one healthy man, carried out five times in the course of three months, showed slight variation — from 6.6 to 12.7% of the total amount of serum vitamin.

The results of the study of patients with chronic myelosis are given in Table 2. In the case of chronic myeloleucosis which is characterized, as is known, by a sharp increase in vitamin B_{12} content in the serum, the absolute amount of "free" vitamin in all cases was considerably higher than in the healthy individuals, but its percent relative to the total amount of vitamin did not have a constant tendency either to be raised or decreased; in three cases it was markedly lower, and in two cases, somewhat higher than the average values found for healthy individuals. The variation in relative amount of "free" vitamin (0-35% of the total amount) in this group of patients did not exceed the limits of variation of it in the blood serum of healthy individuals (0-45%). In the other five hematological patients, in whom the total amount of vitamin was normal, the percent of "free" vitamin also varied within the above limits.

SUMMARY

We have worked out a new method for determination of "free" vitamin B_{12} in blood serum with B. coli 113-3, based on its removal by aqueous acetone with simultaneous

precipitation of the protein. The amount of "free" vitamin in the serum of healthy individuals (28 primary donors) averaged 11.7% of the total amount of vitamin with variation from 0 to 45%. The percent of "free" vitamin with respect to total amount of vitamin B_{12} in the serum of ten hematological patients (chronic myelosis, pernicious anemia in the remission stage) shows practically no difference from the corresponding figures for healthy individuals. In all cases of chronic myeloleucosis the absolute amount of "free" vitamin B_{12} was markedly raised.

We express our thanks to Prof. V. N. Bukin for donating the cultures of B. coli.

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THE SYNTHESIS OF AMINO ACIDS FROM KETO ACIDS AND AMMONIA IN RAT KIDNEY

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Translated from Biokhimiya Vol. 25, No. 1, pp. 86-89, January-February, 1960

Original article submitted June 8, 1959

It was shown by Berezovskaya [1] that the enzymatic system of the rat liver, which catalyzed alanine synthesis from pyruvate and ammonia, was localized in the liver cell mitochondria and that the nuclei of these cells contained an unknown factor which activated this synthetic system.

Berezovskaya [2] isolated this enzyme system by starch-block electrophoresis of extracts from repeatedly frozen-thawed mitochondria, and investigated some of its properties. Since amino acid synthesis from keto acids also proceeds actively in the kidney, it was of interest to establish whether the catalytic system of the latter tissue had similar properties to that of the liver.

METHODS

Kidneys were obtained from adult rats weighing 150-200 g. The capsules, sinuses and most of the medullas were removed and the remaining tissue was homogenized in 9 volumes of 0.44 M sucrose in KCl phosphate buffer (pH 7.4). The suspension was fractionated by differential centrifugation in the cold according to a procedure described previously [3].

Nuclei were sedimented in 10 min at 800 rpm, mitochondria were sedimented from the resulting supernatant in 10 min at 1,200 rpm, and centrifugation of the mitochondrial supernatant for one hour at 1,600-1,800 rpm brought down microsomes.

All fractions were washed with 0.25 M sucrose and resuspended in 0.85% KCl-phosphate buffer. All procedures were carried out in the cold and as rapidly as possible.

Samples of the original crude homogenate and of the various fractions were incubated in the presence of either pyruvate of α-ketoglutarate (100 μM per test) and of ammonium carbonate (final concentration 0.02 M), on the water bath at 38° for 1-2 hours, after which time they were fixed with trichloroacetic acid. ATP (0.001 M) and $MgCl_2$ (0.0001 M) were added to some reaction mixtures. The total volume of the reaction mixture was 3 ml. Amino acids were estimated according to Cocking and Jem [4] and activity was expressed as μM amino acid formed per mg nitrogen of the given fraction.

RESULTS

The results obtained (Table 1) show that amino acid synthesis from pyruvate and ammonia took place in all the fractions examined, but most actively so in the microsomes and mitochondria. The localization of alanine synthesis from pyruvate and ammonia in the kidney thus differs from the liver, in which tissue these reactions proceed practically exclusively in the mitochondria. It is also of interest that in the kidney the synthesis in the mitochondria and the microsomes proceeded at approximately equal rates.

In the kidney nuclei the reaction proceeded somewhat more intensively than in the liver nuclei. It was considered possible that this activity in the nuclear fraction could have been due to contamination by other cellular fragments, debris and whole cells which may have been cosedimented with the nuclei, and which carried the assayed activity. In order to eliminate this possibility the nuclear fraction was further purified according to Schneider and Hogeboom [5]. The purified nuclear fraction was shown to possess a higher specific activity than the crude sediment, and it was therefore concluded that the assayed activity was connected with the true nuclear fraction.

Kidney cells nuclei, as distinct from liver nuclei, produced no stimulation of synthesis whatever in either mitochondria, or in any other fraction investigated. This is seen from the data in Table 2.

When the homogenate was "reconstituted" by a combination of all the derived fractions in correct proportions, the resultant recombined suspension synthesized amino acids at the same rate as the orginal crude homogenate. This is shown in Table 3.

It should be noted that the level of activity in the "reconstituted" homogenate was the same as in a sample of the fresh homogenate which was allowed to stand in the refrigerator for the duration of time required to carry out the complete fractionation procedure and the subsequent reconstitution. Compared with a freshly prepared and not aged homogenate, the "reconstituted" homogenate showed, as a rule, considerably lower activity. This indicated that the synthetic ability of homogenates decreased steadily with aging (4-5 hours).

Incubations in the presence of ketoacids other than pyruvic, namely, with α-ketoglutarate and oxaloacetate, showed that amino acid synthesis in these cases proceeded in the kidney at approximately the same rates as with pyruvate (Table 4).

TABLE 1. Amino Acid Synthesis from Ketoacids and Ammonia in Various Subcellular Fractions of the Rat Kidney (increase in amino nitrogen in μmoles/mg of tissue nitrogen against control incubations fixed with trichloroacetic acid at zero time)

Crude homogenate	Fractions			Superantant
	nuclei	mitochondria	microsomes	
1,8	0,89	1,12	—	—
0,83	0,56	0,68	—	—
0,79	0,49	0,77	—	—
1,06	—	0,7	0,86	0,3
0,88	0,34	0,6	—	—
—	0,56	0,53	0,44	0,4
1,02	0,56	0,61	0,9	0,41
—	—	0,57	1,64	0,24
—	0,54	0,69	—	—
—	0,57	0,78	0,74	0,49
1,04	—	—	0,78	0,49
1,48	—	1,16	0,7	0,82
Mean 1,11	0,56	0,75	0,86	0,44

TABLE 2. The Effect of Combination of Various Subcellular Fractions on the Rate of Alanine Synthesis from Pyruvate and Ammonia (calculation as in Table 1)

Crude homogenate	Nuclei	Mitochondria	Microsomes+ supernatant	Nuclei + + mitochondria	Nuclei + microsomes+ + supernatant	Mitochondria + microsomes + supernatant
1,8	0,89	1,12	1,1	1,2	—	—
1,37	0,17	1,27	0,88	1,23	—	1,29
—	0,94	0,95	—	1,1	—	—
—	0,56	0,53	0,69	0,52	—	0,66
0,79	0,5	0,77	0,43	—	0,57	—
1,06	0,55	0,7	—	—	0,53	—
—	0,57	0,78	0,76	0,7	0,7	0,64

TABLE 3. Alanine Synthesis from Pyruvate and Ammonia in the Original and "Reconstituted" Homogenates (for calculations see Table 1)

Original fresh homogenate	"Reconstituted" homogenate	Original homogenate after standing in refrigerator
0.83	0.58	0.52
0.79	0.66	—
1.06	0.59	0.71
0.88	0.7	0.66
—	0.75	—

Investigation of the effect of added ATP or Mg^{++} on the intensity of amino acid synthesis showed that, as was the case with liver homogenates, these additions had no appreciable effect on the intensity of synthesis. The intensity of this process was also unaffected by the provision of an air or oxygen atmosphere. In this respect the enzymatic system from the kidney behaved identically with the corresponding liver system.

A considerable difference in the reactions under investigation between liver and kidney was revealed when amino acid synthesis was investigated in disrupted mitochondria. Disruption of liver mitochondria by repeated alternative freezing and thawing caused the almost entire activity to pass into the soluble supernatant, while the sedimented mitochondrial fragments showed no detectable activity. Similar treatment of the kidney mitochondria brought about the solubilization of but a portion of the activity; the remainder of the activity could not be separated from the sedimentable mitochondrial fragments and in some cases, as seen from Table 5, the residual activity in the sediment was higher than that in the solubilized fraction.

DISCUSSION

The present results thus show that the main differences between the liver and kidney systems for amino acid synthesis from keto acids rest in the localization of this process in the intracellular fractions and in the degree to which this system is bound by the mitochondria.

It is difficult to ascertain at present the physiological importance of the fact that in the kidney, as distinct from

TABLE 4. Amino Nitrogen Synthesis from Ammonia and Various Ketoacids by Fresh Rat Kidney Homogenates (calculations as in Table 1)

Acids		
Pyruvate	α-ketoglutarate	Oxaloacetate
47.75	63.0	–
34.1	44.35	30.1
37.0	49.0	39.5

TABLE 5. Amino Nitrogen Synthesis from Ketoacids by Whole Mitochondria and also by the Solubilized and Nonsolubilized Mitochondrial Fractions Obtained by Repeated Alternate Freezing and Thawing (calculations as in Table 1)

Whole mitochondria	Solubilized mitochondrial fraction	Sedimented fraction
–	0.34	0.58
–	0.76	–
–	0.6	0.63
0.57	1.0	0.61
0.62	1.5	0.36
0.7	–	0.5
0.78	0.57	0.43
0.54	0.17	0.38
–	0.8	–

the liver, the synthesis of amino acids takes place in all fractions and is not concentrated in the mitochondria alone. It is however of interest that the reverse process (amino acid deamination) in the kidney tissue also occurs in all cellular fractions (unpublished observation).

The fact that the synthetic activity investigated was found to be much more strongly bound to the mitochondria in the kidney than in the liver is apparently also of some physiological importance. Because of this it is considerably more difficult to obtain a soluble purified preparation of this enzyme system from the kidney than it is from liver mitochondria. Work on the isolation of such a purified system from the supernate and from microsomes is at present in progress.

SUMMARY

Amino nitrogen synthesis from ketoacids and ammonia proceeds in all subcellular fractions of the rat kidney: in the nuclei, mitochondria, microsomes and the supernatant fraction. In this respect kidney cells tissue differs greatly from liver, where this synthetic system is localized mainly in the mitochondria.

The intensity of amino nitrogen synthesis was found to be the highest in the mitochondrial and microsomal fraction. Disruption of mitochondria by repeated alternative freeing and thawing caused a partial solubilization of the synthetic system, but considerable residual activity remained bound to the mitochondrial fragments. The binding of this synthetic system by kidney mitochondria is thus considerably stronger than by liver mitochondria, whence it could be easily solubilized by mitochondrial disruption.

The author wishes to thank Prof. S. Ya. Kaplanskii for valuable advise and interest in this work.

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PHOSPHORYLATING AND NONPHOSPHORYLATING PATHWAYS OF OXIDATION. REPEATED "SWITCHING OVER"

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Translated from Biokhimiya Vol. 25, No. 1, pp. 90-95, January-February, 1960

Original article submitted June 10, 1959

About 30 years ago Éngel'gardt observed the formation of ATP in the respiration of nucleated erythrocytes of the pigeon [1]. This discovery was the basis for the study of the coupling of oxidation with phosphorylation. In 1939 Belitser and Tsybakova [2] demonstrated phosphorylation in the transfer of hydrogen by dehydrogenase from the substrate to oxygen with enzymes of the respiratory cycle. This phosphorylation in the respiratory cycle, later called mediator phosphorylation by Shapot [3], was absent after the production of the main mass of ATP in the aerobic cell and therefore was most intensively studied from the point of view of the number, place, and ability to form energy-rich phosphate bonds (~P) in the phosphorylative transfer of electrons and protons in the respiratory chain. However, in studying this question it was frequently observed that systems of biological oxidation in experiments by different investigators and under different conditions showed the ability for oxidation in respiratory chains that was not coupled with phosphorylation. Belitser and Tsybakova [2] noted this fact and called oxidative phosphorylation a "conditionally" coupled process (as distinct from the "unconditionally" coupled glycycolysis). The observation of the ability of intact mitochondria to oxidize in two ways was of great value in the question of nonphosphorylative oxidation (NO): the paths of phosphorylation and those which did not go by phosphorylation [4]. These investigations were continued [5-14] and led to the importance of explaining whether a change in the degree of coupling of oxidation and phosphorylation was a phenomenon of physiological regulation or a defect of it [15]. If the path of nonphosphorylative oxidation occurred as a result of irreversible destruction of the mechanism of coupled oxidation and phosphorylation, then it would be difficult to assume that a share of NO could have regulatory significance If the coupled mechanism could be reversibly detached from the respiratory chain, so that phosphorylative oxidation (PO) could then be connected again, then it would be possible to speak of the regulatory nature of the change in degree of coupling of oxidation and phosphorylation.

Experiments on reversible separation of oxidation and phosphorylation were carried out by Lindberg, Ernster, and co-workers [9, 10, 20] on rat liver mitochondria. PO was switched to NO by preincubation of the mitochondria with glucose and hexokinase in the absence of an oxidation substrate in a medium with Ca or without Mg. The degree of coupling was then again increased by adding Mg (Mn) and ATP or sometimes only ATP.

In the present work we have tried repeated switching over by oxidation in the same preparation of mitochondria from pigeon livers, using a change in ATP content in the system.

MATERIALS AND METHODS

Pigeon livers were homogenized in glass vessel with a Teflon pestle. Mitochondria were separated in 0.25 M sucrose according to the description of Lardy and Wellman [16], suitably simplified for the study of oxidative phosphorylation by the method of Hogeboom, Schneider, and Palade. Oxidation of succinic acid was measured manometrically by oxygen utilization. Phosphorylation was studied by loss in inorganic P which was determined by the method of Lowry and Lopez [17] in a modification which included the addition of copper ($0.8 \cdot 10^{-5}$ M) to hasten the development of the color.*

Method of determining P. To 2 ml of incubated sample which contained 40μM of inorganic phosphate we added 6 ml of 3.3% trichloroacetic acid and then filtered; we collected0.1 ml of filtrate and added to it 5 ml of 0.1 M acetate buffer, pH 4, 0.5 ml $(NH_4)_2MoO_4$ in 0.05 M H_2SO_4, 0.5 ml of 1% ascorbic acid, and a freshly prepared 10^{-4} M solution of $CuSO_4$; the mixture was stirred, allowed to stand 10 min and analyzed colorimetrically. The color was stable for many hours.

The preparation of hexoses was obtained according to Berger et al. [19] up to the fifth stage followed by 12-hour dialysis against a 1% glucose solution. The preparation was kept for months at −15°. The preparations of AMP and ATP were from the Sigma Chemical Company. Reagents for the reaction mixture and for the determination of P were prepared in twice distilled water.

Reaction mixture. Each sample (volume 2 ml) contained: 1) 40 μM potassium phosphate, pH 7.4; 2) 100 μM KCl; 3) 150 μM glucose; 4) hexokinase (threefold excess over the amount which assures maximum rate of phosphorylation); 5) mitochondria from 300-350 mg of liver of 0.25 M sucrose; 6) 50 μM potassium succinate, pH 7.4; 7) 10 μM $MgCl_2$; 8) 3 micromoles AMP.

* A similar modification was used by Peel and Loughman who have recently published detailed work on this question [18].

The first five components composed the preincubation mixture (PM) and the others were added at the moment of beginning the incubation.

Course of Experiments

A suspension of mitochondria was added to the solution which contained phosphate, KCl, glucose, and hexokinase in such amount that in 1.6 ml of PM these components were present in an amount needed for one experiment. The PM was usually prepared for 15 samples (24 ml) and shaken in an Erlenmeyer flask at 26°. At definite time intervals 1.6 ml portions were taken from the flask; these were placed in a Warburg vessel and mixed (at the moment which determined the beginning of incubation) with 0.4 ml of the solution which contained succinate, $MgCl_2$, and AMP (this solution was added from the side). Incubation (oxidation of succinic acid) lasted 20 min. The gas phase was air, temperature 26°. Incubation was ended by adding trichloroacetic acid.

RESULTS AND DISCUSSION

For a demonstration of the possibility of repeated switching over of the oxidative pathways we regulated the degree of coupling by changing the amount of ATP in the system.

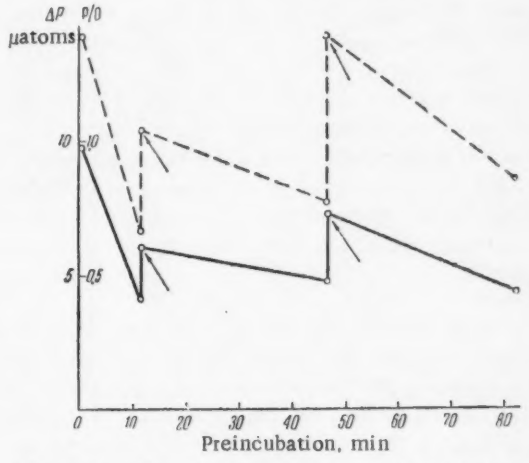
We reproduced the picture obtained by Ernster and Löw with rat liver mitochondria [20] by using pigeon liver mitochondria: changing from PO to NO by preincubation with glucose-hexokinase without substrate and MG and switching again to PO by addition of ATP after preincubation (Table 1). The following stages of the work consisted in repeated uncoupling of the coupling which had been established which occurred by preincubation with hexokinase after adding ATP. New addition of ATP to such a second uncoupled system again (for the second time) increased PO, which was the same as after the first addition of ATP, and sometimes even was greater. Preincubation with hexokinase after the second addition of ATP again uncoupled the PO. The results of typical experiments are shown in the figure.

In this experiment different portions were taken from the flask with the PM as follows. Sample 1 was taken immediately after addition of mitochondria to the stable components of the PM; sample 2 after 11 min of preincubation, after which ATP was added to the flask with the PM to a final concentration of 0.001 M and sample 3 was taken. Sample 4 was taken at 47 min preincubation, then ATP was added to the flask with the PM (final concentration 0.001 M) and sample 5 was taken. After 36 min more, sample 6 was collected. In all the samples after collection we added a mixture consisting of succinate, $MgCl_2$, and AMP and then incubated for 20 min. The samples which had been preincubated were introduced into the vessel in such a way that the time of preincubation (complete or partial) was used for equalizing the temperature. Under these conditions the absorption of oxygen (ΔO) was measured without extrapolation. Calculation of ΔO in the samples without preincubation was carried out with extrapolation to 10 min of equalizing the temperature.

TABLE 1. Switching the Oxidative Pathway by Preincubation with Hexokinase and Addition of ATP after Preincubation

P/O before preincubation	P/O after preincubation	
	without ATP	With ATP
1.3*	0.75	1.35
0.95	0.7	0.9
1.25	0.85	1.15
1.4	0.6	0.95

* In this experiment the concentration of ATP was 2 μM/sample; in the remaining it was 1 μM/sample.



Change in value of phosphorylation and coefficient P/O in five-fold switching of the oxidative pathway. Dotted line) ΔP; solid line) P/O; arrow indicates moment of addition of ATP.

As the figure shows, we could follow the change of six different ratios of PO and NO depending on ATP content in the system and fivefold switching of the oxidative pathway: The initial system (sample 1) was characterized by a definite PO level which fell sharply (sample 2) with rise in the share of NO as a result of preincubation with hexokinase (first switch); in sample 3 the addition of ATP to the system raised the share of PO (second switch); further preincubation with hexokinase led to exhaustion of the added ATP and rise in NO (sample 4, third switch); in sample 5 addition of ATP again raised the share of PO (fourth switch); finally, further preincubation with hexokinase again switched the transfer of electrons to NO (sample 6, fifth switch).

An analogous change of state is shown by the data of Table 2. The final concentration of added ATP in the experiments (Table 2) was 0.001 M. It is interesting that the first switch to NO was often not fully reversed by addition of ATP (which confirms the findings of Ernster on rat liver mitochondria [20]). Repeated switching to NO (after the first addition of ATP and following preincubation) was always completely reversed by new addition of ATP.

Probably the first switch to NO can be related not only to exhaustion of endogenous high-energy bond reserves in the mitochondria, but also to some other changes which are not related to the ATP content. Repeated switching to NO is caused by utilization of added ATP and therefore is fully reversed by the reduction in concentration of this substance in the system.

The purpose of these experiments was to demonstrate the possibility of repeated switches in the oxidative pathway and did not try to show the maximum number of switches which could be obtained with one preparation of mitochondria using this method. We can only observe that according to the experimental data, five switches are not the limit. By lengthening the time of preincubabion we were able to show 6 and 7 switches (third addition of ATP and following preincubabion). However, here there was a supplementary difficulty because of the occurence of secondary effects connected with the fall in rate of oxidation with long preincubation (Table 2, experiment II). In a number of experiments ATP added before preincubation raised P/O. Thus, 8 switches, besides the methodological difficulties, were connected with decreased amplitude of variation of P/O. For these reasons, switching the oxidative pathway more than five times has not been described in detail.

The switch in oxidative pathway by change in ATP content is a convenient model of repeated switchings over: Endogenous ATP evidently is exhausted by preincubation with hexokinase (since this preincubation causes uncoupling and converts ATP), and ATP added in catalytic amounts is also used up rapidly enough by hexokinase and the adenosintriphosphatase system of the mitochondria. Thus there exists the possibility of decreasing the ATP content and reducing the amount added to the medium, that is, of changing the ratio of the pathways by one factor.

Since this factor, which "switches over" the oxidative pathway, is ATP, which also has a role in the enzymatic properties of the mitochondria [9, 10, 20-23], it is necessary to determine the place which ATP can occupy in the functional scheme of regulating the oxidative pathway on the basis of our experimental data and the materials published in the literature. In our experiments, stimulation of PO by ATP is not connected with increased acceptor ability of the adenylic system in the reaction mixture, since in all the samples, independent of the addition of ATP, the AMP content was sufficient to reach maximum rate of transfer of the ~ P which was formed to glucose, and in the absence of hexokinase AMP was quantitatively transformed into ATP. AMP cannot replace ATP in the function of "switching over", although there are data indicating that AMP can raise the concentration of intramitochondrial ADP to a greater degree than can ATP [29]. The ineffectiveness of ADP in restoring coupling was shown in the experiments of Ernster and Löw [20]. These results lead to the conclusion that the function of stimulation of PO is specific for ATP. Thus it is shown that ATP is not only a product of PO but is also a necessary condition for mitochondria to undergo phosphorylative oxidation.

However, regulation of the oxidative path only by changes in the content of ATP could scarcely occur in vivo, in wide limits in all cases in the normal: Mitochondria impoverished in ATP do not phosphorylate and so are not in a state to synthesize ATP for switching their PO; on the other hand, phosphorylating mitochondria produce ATP and for this reason cannot switch to NO without the intervention of some other agents. In order to realize the switching over by ATP supplementary factors are needed which make this type of regulation possible. In pigeons such factors are evidently activated in the change from the winter type of metabolism to the spring-summer type which is accompanied by intensive molting (end of February to April). In just this period preincubation of the mitochondria without oxidation substrate with hexokinase (or even without hexokinase) even after 10-20 min leads to marked switching over to NO (figure and Table 2), while in winter, preincubation even with 10-20 times excess of hexokinase cannot lower P/O. It is characteristic that the initial level of P/O (before preincubation) during the period of labilization of switching over is decreased (1-1.4 times instead of 1.5-2 in the winter series of experiments).

TABLE 2. Repeated Switching of the Oxidative Pathway

Preincubation, min	Time of adding ATP, min	Δ O, μatoms	Δ P, μatoms	P/O
		Experiment I		
—	—	16,5	18,5	1,12
15	—	17,2	6,3	0,36
15	15	17,0	9,5	0,56
50	15	18,5	6,2	0,34
50	15 and 50	16,0	10,0	0,63
		Experiment II		
—	—	12,0	13,5	1,15
25	—	12,0	8,0	0,67
25	25	11,7	11,0	0,94
95	25 & 65	4,8	4,0	0,83
95	25, 65 & 95	5,5	6,5	1,15
140	25, 65 & 95	3,0	0,0	0,00

The uncoupling of PO by preincubation with hexokinase in the experiments of Ernster on rat liver mitochondria developed very quickly. As a model for repeated switchings this material could hardly be as convenient as pigeon liver mitochondria, since Ernster showed a very rapid loss of the effect of stimulation of PO by ATP during preincubation of rat mitochondria. In the same time in pigeon liver mitochondria the effect of ATP is evidently more stable: We obtained a P/O greater than 1 in the oxidation of succinic acid by mitochondria preincubated 3.5 hours at 26° when ATP was added to the medium each 30 min of preincubation.

The demonstration of the possibility of repeated switching over of oxidative pathway experimentally confirms the view that switching pathways is a reversible regulation of the degree of coupling of oxidation and phosphorylation. This fact can be established in connection with the evidence for the biological justification of the variability in value of P/O for thermoregulation [24, 25], direction of synthetic metabolism [26, 27], or reaching maximum rate of producing ATP [28] and the evolution of P/O in a series of vertebrates [13]. This opens prospects for a study of the participation of phosphorylative and nonphosphorylative oxidative pathways in the functional scheme of metabolism.

SUMMARY

We have demonstrated the practicality of switching over of phosphorylative and nonphosphorylative pathways of oxidation in the same preparation of pigeon liver mitochondria by change in the content of ATP in the system. The uncoupling of oxidation and phosphorylation is attained by preincubation of the mitochondria with hexokinase and glucose in the absence of oxidation substrate (succinic acid) and the restoration of coupling of oxidation and phosphorylation by addition of catalytic amounts of ATP.

The fact of repeated switching over of the phosphorylative and nonphosphorylative oxidative pathways is considered as evidence that change in the degree of coupling of oxidation and phosphorylation can be of a regulatory nature and can take part in regulating metabolism.

We express thanks to Sergei Evgen'evich Severin for his many valuable suggestions and critical comments in carrying out this work and preparing it for the press.

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THE NUCLEOPROTEINS OF NORMAL AND REGENERATING LIVER

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Translated from Biokhimiya Vol. 25, No. 1, pp. 96-101, January-February, 1960
Original article submitted June 17, 1959

In a previous paper [1] we described the development of our method of "temperature" fractionation of nucleoproteins (NP) separated from beef and rabbit livers. Fractionation was carried out at 0, 20, 37° in 0.5 M NaCl solution which contained a deproteinizing agent, a mixture of chloroform and butyl alcohol. We thus obtained NP fractions which clearly differed from each other in ratio of nitrogenous bases.

In the present work will develop the problem of whether there is a difference in ratio of nitrogenous bases in NP fractions of normal and regenerating tissues. According to the literature data, such differences do not exist [2]. However, such results may be due to the fact that whole unfractionated NP was analyzed, and the slight differences which existed might not have been noticed. Also, we wish to determine whether there are differences in biological properties of NP fractions from normal and regenerating liver.

EXPERIMENTAL

To obtain a nucleoproteins we used the livers of male rabbits weighing 2-2.5 kg. Usually for each experiment we took the livers from ten animals. In order to obtain regenerating livers, we first removed 2/3 of the organ, always the right and left lobes, with application of ligatures at their base.* The separated parts of the livers were washed with a 0.14 M NaCl solution and rapidly frozen with dry ice. The rabbits were killed 68-70 hours after hepatectomy. We chose this period of regeneration because there is considerable mitosis in rabbits' liver cells 72 hours after hepatectomy [3, 4]. The regenerating liver was treated in the same way. We obtained deoxyribonucleoprotein from the samples of normal and regenerating liver by the method of Mirsky and Pollister [5]. We determined the DNA content of the whole nucleoprotein by the Fiske-Subbarow method, the nitrogen content by Kjeldahl-Conway method, the molecular weight of nucleoprotein and DNA in the nucleoprotein by the method of Spitkovskii [6], and the nitrogenous bases by chromatography by the Spirin modification [8] of the Wyatt method [7]. For fractionation we used nucleoproteins with a N/P ratio of 3.7 : 3.8 which was obtained by triple reprecipitation of the nucleoprotein from six volumes of distilled water. The initial molecular weight of DNA in the nucleoprotein was 4-4.5 million. Fractionation of whole RNP was carried out 3 days after fractionation of whole NNP, that is, with the same interval as in the isolation of NP.†

Fractionation was carried out by the method described elsewhere [1]. After separation of the three fractions of NP, the remaining portion had the form of a dense film and consisted chiefly of protein; it was discarded. In the fractions which we obtained we determined the content of DNA and nitrogen, the molecular weight of DNA and the ratio of nitrogenous bases by the methods mentioned above. In all, we fractionated six NP samples. Three of these were normal liver and three regenerating. In each experiment we made two parallel, independent fractions, and the results were close to each other (Tables 1 and 2).

When we compare the results of fractionation of NP from normal and regenerating livers (Table 1) we see that the first fraction from RNP is somewhat smaller, and the second and third fractions somewhat larger than the corresponding fractions of NNP. In both cases the first fraction makes up more than two thirds of all the NP. The same conclusion can be drawn with respect to the DNA content in each fraction. Our method of isolating and reprecipitating NP gives well-reproducible results. The value of N/P in all the NP isolated from both normal and regenerating livers is always about 3.7-3.8. Change of N/P in fractionation occurs to about the same extent. The value of N/P in the first fraction is close to the value of N/P in the starting nucleoprotein. In the second fraction the value of N/P decreases, that is, the relative amount of DNA rises. In the third draction the value of N/P again rises sharply, which shows the presence of a considerable amount of protein, perhaps not even bound to DNA. This increase is considerably greater in the case of RNP than in the case of NNP.

We should mention that the use of our fractionation method has little effect on the degree of polymerization of DNA, as is clear from the values for the molecular weight given in Table 1. The criterion for the accuracy of the determination is the ratio of purines to pyrimidines, which according to the Chargaff rule should be close to

* We thank Candidate in Medical Science N. S. Artem'eva for carrying out the hepatectomies.

† Designation: deoxyribonucleoprotein from normal liver – NNP, from regenerating liver – RNP.

TABLE 1. Characteristics of NP of Normal and Regenerating Livers

Substance	Undamaged liver					Regenerating liver				
	% of whole NP	DNA (mg in fraction)	N (mg in fraction)	N/P	mol. wt. of DNA, million	% of whole NP	DNA (mg in fraction)	N (mg in fraction)	N/P	mol wt of DNA, million
Whole NP in 1 M NaCl	100,0	62,0	22,25	3,70	4,0	100,0	62,0	22,65	3,78	4,9
First Fractionation										
Fraction I	70,0	43,56	15,2	3,60	3,5	69,0	42,64	15,1	3,64	4,2
Fraction II	10,5	6,54	1,97	3,10	3,8	14,5	8,84	2,6	3,09	4,1
Fraction III	8,0	4,98	1,94	4,01	3,2	11,0	6,95	3,2	4,70	3,2
Residue	11,5	—	—	—	—	5,5	—	—	—	—
Second Fraction										
Fraction I	75,0	52,93	17,9	3,48	3,4	67,0	47,58	15,9	3,44	4,3
Fraction II	11,0	6,67	1,90	2,94	3,6	13,0	10,86	3,06	2,89	4,6
Fraction III	7,0	2,35	1,07	4,25	2,9	6,0	3,24	1,6	5,07	—
Residue	7,0	—	—	—	—	14,0	—	—	—	3,3

TABLE 2. Ratio of Nitrogenous Bases in NP Fractions of Undamaged and Regenerating Liver*

Substance	Undamaged liver				Regenerating liver			
	A/T	G/C	purine / pyrimidine	A + T / G + C	A/T	G/C	purine / pyrimidine	A+T/G+C
Whole nucleoprotein	0,98	1,08	1,02	1,19	0,99	1,13	1,06	1,13
Fraction I	0,96	1,03	0,99	1,20	1,02	1,00	1,01	1,36
Fraction II	1,04	1,05	1,05	0,99	0,99	1,06	1,02	1,04
Fraction III	1,10	1,08	1,09	0,85	0,98	1,05	1,01	0,82

*A) Adenine, G) guanine, T) thymine, C) cytosine.

unity. As Table 2 shows, in all the fractions the variation from unity in the ratio of purines to pyrimidines is slight.

The value of the specificity factor A + T/G + C for whole NP from normal and regenerating livers is practically the same. The second and third fractions of nucleoprotein also differ little from each other in this index. On the other hand, there is quite a sharp difference in the specificity factor in the first fraction: 1.20 for NP from normal liver and 1.35 from regenerating liver. Evidently the regenerating liver contains DNA or nucleoproteins which differ in the composition of nitrogeneous bases from the DNA or nucleoproteins of normal liver.

We carried out special experiments to test the biological activity of NP and its fractions isolated from regenerating and undamaged liver of rabbits.

The first experiments, which had a preliminary character, were carried out on tadpoles of grass frogs (Rana temporaria) in stages II and IIIa of their development. We removed about a third of the liver through a cut in the side of the animals which was made with ophthalmic scissors. The wound was not sutured since healing occurred very quickly. Mortality was low. On the day after the operation the tadpoles were injected intraperitoneally with the corresponding solution. Injection was carried out with a syringe, introducing the needle through the dorsal muscle by the method described by Pukhal'skaya [9]. One group of tadpoles received Fraction I from regenerating liver of rabbits, a second group the analogous fraction from undamaged liver, and a third a salt solution of the same concentration as the nucleoprotein fractions. The amount of fraction injected in all cases was equalized as to DNA and protein.

The main difficulty with the plan of these experiments was the high mortality of the tadpoles caused by the injection of the hypertonic salt solution; therefore we operated on a large number of tadpoles, of which only a small number survived. The tadpoles were killed two days after the operation, when the whole liver was again nearly normal. There were three groups of animals: controls, those receiving salt solution, and those receiving fraction I of NNP; the weights of their livers were the same. (The differences between them were not statistically significant.) On the other hand, in the tadpoles which received fraction I and of RNP the weight of the livers was greater, exceeding the statistical error ($p = 0.000$). The difference in weight of the livers of the first two groups

and the livers of tadpoles injected with RNP was about 20%. The experiment was repeated three times, and in all cases we found a greater weight in the livers of tadpoles which received RNP.

After these preliminary experiments which showed that the nucleoprotein fraction from regenerating liver of rabbits could act on the regenerating liver of animals of another vertebrate class, we ran the main experiments using mice as the experimental animals.

The experiments were carried out on 95 mice, weighing 18-20 g. We removed the left lobe of the liver in all the mice. The weight of the removed lobe averaged 323 mg, that is, about one third the weight of the whole liver. The mice were divided into three groups. The first group (50 animals) was injected in the caudal vein with 0.1 ml of a solution of fraction I of NNP which contained 17 mg of DNA. The second group (29 mice) was injected with the same amount of a solution of fraction I of RNP. The animals of the third group (16 mice) were injected with a salt solution of the same concentration as those which contained the nucleoprotein fractions. After four days the animals were killed. The relative weight of the livers of the mice of the group which had been injected with the fraction from RNP was about 15% greater (4.98 compared to 4.30). The difference between the two groups was statistically significant ($p = 0.000...$). The weight of the livers of the mice which were injected with salt solution was almost the same as the weight of the livers of the mice injected with NNP solution (4.39 and 4.30 respectively). The differences in this case had a purely chance character.

A special series of experiments was undertaken to determine the biological activity of the different fractions of isolated nucleoproteins from the regenerating rabbit livers. The mice were divided into four groups of 11 animals. Mice were injected with salt solution (group 1) and with solutions of fractions I, II, and III from RNP in the same amounts as in the previous series of experiments. The injection of the solutions as in the previous series was carried out on the day following the operation and the removal, on the fourth day after the operation. The weight of the mouse livers of groups 3 and 4, did not differ significantly from the weight of the livers of mice from group 1, while the weight of the livers of the animals in group 2 was 10% higher (the difference was close to statistical significance, $p = 0.045$). Thus only Fraction I of RNP shows an effect on regenerating livers of mice.

As a large amount of work has shown, using different mammals, mainly rats, regeneration of livers with one or more lobes removed occurs by a type of regenerative hypertrophy. The weight of the livers is restored quickly (in 1-2 weeks) as a result of an increase in the number of cells due to mitotic multiplication. Mitosis occurs equally in the whole organ. Destructive phenomena in the regenerating liver are weak. The original form of the organ is never restored. In other words, restoration occurs because of formation of new cells within the remaining lobes of the organ and not on the wounded surface.

In order to explain how regeneration of the liver occurred in our experiments and on what the increased weight of regenerated liver of mice which received fraction I of RNP depended, we carried out a morphological analysis. The mouse livers were fixed in Zenker's solution and pieces of different parts of the liver were embedded in paraffin, cut into sections 8 μ thick, and stained with hematoxylin-eosin. In order to compare the rate of multiplication of the mouse liver cells for the different groups, we counted the mitosis in livers of mice which had received the fraction from RNP and in livers from mice which had received the fraction from NNP. Mitosis was counted in 300 visual fields with ocular 7, immersion objective 90 and a square aperture in the ocular diaphragm equal to 7 × 7 mm. The count showed that using this method we could determine the amount of mitosis in an average of 6000 cells. There is a daily periodicity of mitosis in regenerating mouse liver cells, as one of our studies has shown [10]. In 10-12 hours, when removal occurred on the fourth day after the operation, mitotic activity should not be high. Our data confirmed this conclusion. In the livers of 21 mice from the group which received RNP we observed 78 mitoses, an average of 3.71 mitoses per mouse or 0.62%. In the livers of 17 mice from the group which received nucleoprotein from unoperated livers we found 57 mitoses. This was 3.35 mitoses per mouse or 0.56%. The difference between the averages-in both groups of experiments was entirely chance, as statistical treatment showed. Thus, four days after the operation, in the mice of the different groups cell multiplication occurred at about the same rate. Finally, we cannot conclude that during the period immediately after the operation, as on the second or third days for example, there was a preponderance of rate of multiplication in the livers of mice which had received nucleoprotein from regenerating liver. However, such an idea is not sufficiently supported. Therefore we can draw the orienting conclusion that the injection of nucleoprotein from regenerating liver has no actual effect on rate of cell multiplication in regenerating liver.

Since we wished to explain whether the observed increase in weight of the liver could be due to greater diameter of the cells, we counted the number of cells in the visual field. The count was made in 50 visual fields in the liver of each mouse. Conditions of counting were the same when counting the number of mitoses. The count was made in the livers of 15 mice which had received RNP and in the livers of 15 mice which had received NNP. The average in the first case was 15.89, and in the second, 13.45. The difference between the averages was statistically significant ($p = 0.005$).

Thus, cells in the liver of mice which had received nucleoproteins from regenerating livers were larger. The difference was about 20%, that is, it reached the same size as the difference in weight of the livers of the two groups of mice. We can therefore assume that the difference in weight is connected with hypertrophy of the cells caused

by injection of nucleoproteins from regenerating liver. We should mention that in agreement with the data of Ryabinina [10] hypertrophy of the cells is characteristic for mouse livers 2-3 days after injury. Evidently, in our experiments this hypertrophy was more obvious.

In order to determine whether the hypertrophy concerned only the body of the cells or their nuclei, we measured the size of the mouse liver nuclei of both the groups being compared. The nuclei were traced on millimeter paper with an Abbe tracing apparatus; the paper was cut out along the projected contours and weighed on a torsion balance. The size of the nuclei was estimated from the weighings. We measured 100 nuclei in each liver. Measurement of nuclei in 10 livers of mice of the first group and from 10 mice of the second showed that there was a difference in the size of the nuclei, namely, they were larger in mice which had been injected with nucleoprotein from the regenerating liver. The average in the latter case was 4.40 and in the first case, 3.79. This difference was close to statistical significance ($p = 0.04$).

Thus, injection of fraction I of the nucleoprotein from regenerating liver not only strengthened the hypertrophy of cell cytoplasm, but also caused hypertrophy of their nuclei.

DISCUSSION

It follows from the preceding material that our idea of the process of "temperature" fractionation permits us to get reproducible results, and the difference in DNA composition of the factor of specifity is sufficiently clear. It is very probable that "temperature" fractionation occurs because of a difference in stability of the protein-DNA bound of different chemical substances to external action. Thus it was recently shown that DNA of the A-T type is less stable to the action of temperature than is the G−C type [11]. In our case, as it happened, the DNA fraction of type A−T separated first, and then, with increased temperature, the G−C type. We also see that the method of comparative fractionations of NP from normal and regenerating rabbit livers shows a sufficiently clear and reproducible difference in the composition of their DNA.

We must mention that the failure, up to now, in attempts to find such differences in the composition of the nucleic acids of organs in different physiological or pathological states is explained by the fact that the comparison of the composition of the nucleic acids was produced without first fractionating them. Unfortunately we can still say nothing about what the difference in DNA composition which we observed depends upon. The idea that it depends upon contamination by RNA which is present in varying amounts in the studied preparations is contradicted by the experiments first carried out. It is possible that the difference results from a comparatively low polymer DNA fraction, the amount of which should be high enough in regenerating liver on account of intensive synthesis of DNA in it.

What actually determines the biological activity of fraction 1 of RNP is also unknown. It differs in the composition of the nitrogenous bases in its DNA from the analogous and biologically inactive fraction 1 of NNP. However, we still cannot relate the biological activity to this difference. It is very likely that this is only an accidental fact, and that actually the biological activity of the nucleoprotein depends on some other factors. We should mention here that the presence in regenerating liver of a particular factor which stimulates regeneration has already been described, but the nature of its action remains unsettled [12]. Finally, the data which we have obtained on the character of the biological action of the fraction has only a preliminary nature. We have used only one dose of injected substance and only one of the possible variant of the experiments, that is, the mice have been killed only four days after the operation. Under these conditions we observed that some of the studied NP has biological activity which is reflected by a strengthening hypertrophy of the cells in the regenerating liver. It is possible that a more detailed experimental plan would also detect the effect of NP on other parts of the regenerating process, which may be indicated by the observed hypertrophy of the nucleus.

SUMMARY

We have presented a method of fractionating deoxyribonucleoproteins by the action of different temperatures which gives good, reproducible results and shows a clear difference in factors of specificity between the separate fractions.

Comparative fractionation of deoxyribonucleoproteins from normal and regenerating rabbit livers has shown that one of the nucleoprotein fractions from regenerating liver differs in its factor of specificity from the corresponding nucleoprotein fraction isolated from normal livers.

Intraperitoneal injection of fraction I of deoxyribonucleoprotein from regenerating rabbit liver into tadpoles of the grass frog showed a stimulating effect on the regeneration of the tadpole liver.

Intraperitoneal injection of the same fraction into mice, part of whose liver had been removed, caused an increased weight of their regenerating livers as compare to the weight of regenerating liver of mice injected with the fraction of normal rabbit liver. Morphological analysis showed that this increase in weight of the mouse livers was basically caused by hypertrophy of the liver cells with increased diameters of both cytoplasm and nuclei.

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THE PREVENTIVE EFFECT OF PROLONGED ADMINISTRATION OF SORBITOL IN CHOLINE INSUFFICIENCY

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Translated from Biokhimiya Vol. 25, No. 1, pp. 102-105, January-February, 1960

Original article submitted June 18, 1959

Choline insufficiency in rats produces characteristic changes in the kidney (so-called "hemorrhagic degeneration") and fatty infiltration of the liver which occurs on the fourth to fifth day of the experiment. Also, in young animals there may follow a spontaneous healing of the kidney, but the degree of fatty infiltration of the liver continues to increase and after five to eight months may lead to cirrhosis and later even to tumors of the liver [1-4].

In a previous communication [5] it was shown that the inclusion of sorbitol for a relatively short time in the experimental choline-free diet as a replacement for sucrose could greatly hinder and even entirely prevent the kidney changes; such a substitution, however, did not prevent the liver changes in which there usually developed fatty infiltration in choline insufficiency. The experiments were so carried out that for eight days the rats received an adequate diet in which sucrose was gradually replaced by sorbitol; from the ninth day the rats began to receive a specially treated choline-free diet in which the carbohydrate has fully replaced by sorbitol. The animals received this diet for ten days. Thus, the preliminary taking of sorbitol for eight days followed by ten days of taking it with the choline-free diet furnished conditions in the organisms which assured synthesis of choline (or its precursors) limited in intensity.

Under these conditions, choline was synthesized in amounts sufficient to hinder development of changes in the kidney but insufficient to prevent fatty infiltration of the liver. In this connection, the question arises whether we cannot prolong the preliminary uptake of sorbitol in the diet to raise the choline synthesis so that it will hinder not only kidney changes, but also fatty infiltration of the liver. The aim of the present work was to solve this question. The results of the experiments showed that prolonging the preliminary intake of sorbitol prevents pathological changes in the liver which could not be prevented by a short period of intake in the previous investigation.

EXPERIMENTAL

We carried out two series of experiments which differed in the preliminary inclusion of sorbitol in the diet.

First series. We used 15 rats, weights 55 g; of these, five controls received the choline-free diet with sucrose. Ten received the same diet with sorbitol; at the beginning of the experiment they were given a supplement of 30 mg of choline hydrochloride per day. Sorbitol was given in gradually increasing amounts to prevent development of diarrhea. The composition of the experimental diet and the dosage of sorbitol have been described elsewhere [5]. In three control rats and in three which received sorbitol, the intestinal flora were studied by systematic culturing of the feces.*

The investigation showed that replacing sucrose in the diet by sorbitol caused a change in the intestinal flora: It sharply decreased the culturing of rod-shaped microorganisms, especially the intestinal bacilli and protei, and also the slime forming microbes and anaerobes; the cocci prevail, especially the enterococci and staphylococci (white and cream colored).

After 74 days, this change in flora in the experimental rats assumes a stable character, and from this time on choline was excluded from the diet. At the same time, five control rats of the same weight as that of the experimental animals began to receive a choline-free diet with sucrose, the composition of which has been described elsewhere [5]; until this time they had received the ordinary mixed diet (grain, meat, bread, milk, vegetables).

Biopsy showed that while the control rats which received sucrose showed great fatty infiltration, the same was not found in rats which received sorbitol. All the rats were killed 27 days after beginning the choline-free diet. Determination of the amount of fat in the liver (ether extraction of the dried liver in the Soxhlet apparatus) confirmed that the inclusion of sorbitol in the diet prevented the development of fatty infiltration (table).

Photomicrographs of the rat liver preparations from the choline-free diet with sucrose and with sorbitol illustrate the difference in histological picture on the 27th day of the choline-free diet (figure).

Second series. In the second series we used 15 rats with an initial weight of 55-65 g. As in the first series, 10 rats received the choline-free diet in which sorbitol replaced the carbohydrate: choline was added to this diet

* This part of the study was carried out in the Department of Microbiology of the Second Moscow Medical Institute by Assistant A. V. Lazgunova.

for 90 days. On the 91st day of the experiment choline was removed from the diet. On this day the controls for the experiment were set up, five more rats which received the choline-free diet. Sorbitol was not included in this diet, and sucrose was the carbohydrate. Both groups of animals received their experimental diets for 23 days. On the 24th day all the animals were killed, and the fat content in the rat livers of both groups was determined (the liver was also studied microscopically).

As in the first series of experiments, the animals which had not first received sorbitol showed microscopically a great fatty infiltration, and the picture was identical with that shown in the figure (A). In the liver cells of animals which received sorbitol no fatty accumulation was found.

Determination of the amount of fat showed:

Choline-free diet	Fat content in liver, %
With sucrose	40.0
	57.4
	62.5
	64.8
With sorbitol	15.4
	15.5
	18.1
	25.0
	25.0
	25.6
	27.8
	33.2
	44.1

DISCUSSION

In the experiments of Morgan and Yudkin [6] the inclusion of sorbitol along with sucrose in the diet hindered the development of B_1 avitaminosis, and also other avitaminoses of the B group; sorbitol also prevented a fall in body weight in animals deprived of choline. As in the experiments published earlier [5], as also in the experiments described in the present communication, it has been shown that the inclusion of sorbitol in the diet instead of carbohydrate yields conditions in the organism which assure endogenous formation of choline. If, however, a relatively short period of sorbitol inclusion in the diet in the first investigations prevented changes in the kidney, but did not prevent fatty intiltration of the liver [5], then the experiments described in this paper with prolonged preliminary inclusion of sorbitol in the diet gave conditions in which later exclusion of choline from the diet did not produce fatty infiltration of the liver. Since the liver is a more sensitive organ than the kidney to lack of choline, this fact indicates that more prolonged use of sorbitol and a greater amount of it assures a more intensive endogenous formation of choline or its precursors. The changes in the intestinal flora which we have established give us a reason for assuming that the preventive effect of sorbitol is connected with the development in the intestine of the new flora which assure a more intensive formation of choline.

TABLE. Content of Fat in Livers of Rats Receiving a Choline-Free Diet with Sorbitol and a Choline-Free Diet with Sucrose

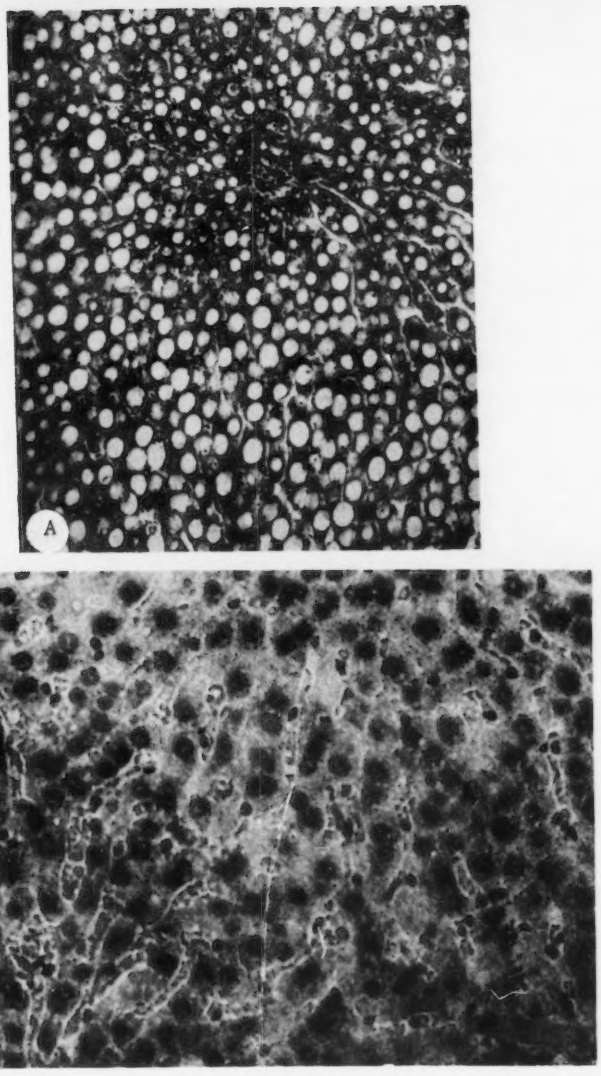
Choline-free diet	Fat content in liver, %
With sucrose	25,0
	63,0
	65,2
	65,6
	70,4
With sorbitol	11,0
	13,4
	13,8
	14,2
	15,8
	16,6
	16,8
	18,6
	18,6
	36,6

However, we cannot exclude the possibility of another method of action of the intestinal flora. The intestinal flora of humans, rats, and also pigs can transform choline into trimethylamine, and this, in the opinion of some authors [7-9] decreases the amount of absorption of choline. Therefore, the preventive effect of new flora can be connected not only with the ability to form choline intensively, but also with the absence of the active ability to transform it to trimethylamine; therefore the choline which passes unchanged with the food can be absorbed in greater degree in the intestine.

Further study should explain which of these two possibilities is actually the reason for the observed preventive effect. However, se should also remember that Riedels and Hines [10] deny the ability of ordinary rat intestinal flora to decrease the amount of choline absorbed. If the results should confirm these authors, then we should most probably assume that in our experiments it is most likely that there is more intensive endogenous synthesis of choline (or its precursors) connected with the observed change in intestinal flora. This question will be solved by decisive experiments. Independent of the mechanism of the preventive effect, our experiments have shown that the inclusion in the diet of sorbitol can prevent the development of serious pathological processes since fatty infiltration of the liver in choline insufficiency is only the first stage which is followed not only by cirrhosis, but often by the development of malignant tumors [2-4], and changes in the kidney lead to development of hypertrophic syndrome [11].

SUMMARY

Prolonged inclusion of sorbitol in choline-free diet instead of sucrose prevents development of pathological changes in the liver. As a result of replacement of carbohydrates in the diet by sorbitol we have observed a change



Prevention by sorbitol of fatty infiltration of the liver in rats receiving a choline-free diet. A) Fatty infiltration in the choline-free diet with sucrose; B) choline-free diet with sorbitol (no fat accumulation in the cells). Stain, Hematoxylin-eosin.

in character of the intestinal flora. There is reason to suppose that the prevention by sorbitol of the development of symptoms of choline insufficiency is connected with just this effect of change in intestinal flora.

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THE EFFECT OF VARIOUS ENZYME INHIBITORS ON THE PURIFIED ENZYMATIC SYSTEM WHICH SYNTHESIZES AMINO ACIDS FROM KETOACIDS AND AMMONIA

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Translated from Biokhimiya Vol. 25, No. 1, pp. 106-111, January-February, 1960

Original article submitted July 1, 1959

We have reported elsewhere [1-3] that rat liver mitochondria contained a transaminase-independent enzymatic system which catalyzed alanine synthesis from pyruvate and ammonia. It was of great interest to establish the sensitivity of this enzymatic system to a variety of enzyme inhibitors and other chemical agents, since information of this kind could aid in the elucidation of the nature of this enzyme and the mechanism of its action.

METHODS

The isolation and purification method (starch electrophoresis of an extract from mitochondria, where this system is localized) has been described in detail in an earlier communication [2]. The enzyme preparation employed in the present work consisted of those enzyme fractions, collected from the starch block, which showed the highest activity (sections nearest to the anode). This active preparation was incubated with pyruvate (80 μM per test) and ammonium carbonate (final concentration 0.02 M) in the presence of various inhibitors (over a range of concentrations). The total volume of the reaction mixtures was 3 ml. ATP and $MgCl_2$ were added to the mixtures to a final concentration of 0.001 M and 0.0001 M, respectively.

Increase in amino-N was determined according to Cocking and calculated as μM/mg N in the reaction mixture. Nitrogen was estimated according to Lowry. In the majority of cases the enzyme preparation was preincubated with the inhibitor at room temperature for 10-15 min before the addition of the pyruvate.

RESULTS AND DISCUSSION

Monobromoacetate and arsenite, employed at a concentration 1×10^{-2} M, inhibited amino-N formation by 65-75%; the inhibition decreased as the inhibitor concentrations decreased (Table 1). The two compounds are very active inhibitors of -SH groups; it could therefore be expected that the activity of the enzyme under investigation depends to some extent on the -SH groups of the enzyme protein.

In the present experiments, cyanide and fluoride showed considerable inhibition (80%) of the increase in amino-N only when present in high concentrations (1×10^{-2} M). This inhibitory effect disappeared completely when the inhibitor concentration was lowered to 1×10^{-3} M. The fact that in the present system KCN was not inhibitory at concentrations (10^{-3} M) where it inhibits respiration, need not be surprising in the light of the finding of Neber [4], and also of the present author, that amino acid synthesis in tissue slices and homogenates was independent of the presence of oxygen.

The results obtained with hydroxylamine and semicarbazide, both reagents for the carboxylic group, are of interest. When present in high concentration (1×10^{-2} M), these compounds inhibited the increase in amino-nitrogen by 70-80%; At lower concentrations (1×10^{-3} M) the inhibition was less pronounced, amounting to only 35-40%. It is possible that these compounds had no direct effect on the enzyme system and acted by combining with pyruvate and removing part of this substrate from the reaction thus preventing its amination.

Braushtein and Azarkh [5, 6] investigated the effect of a number of transamination inhibitors on the process of amino acid synthesis from pyruvate in liver homogenates. They found that hydroxylamine which, at a concentration 4×10^{-3} M, exhibited pronounced inhibition on transamination, also inhibited amino acid synthesis from pyruvate. In accordance with the general concepts forwarded by these workers, they assumed this inhibition to be due to suppression of transaminase. The results with the present purified enzyme preparation, free from transaminase activity, suggest that hydroxylamine also exerts inhibition on the system catalyzing the direct synthesis of alanine from pyruvate and ammonia. The same also holds for cocaine. The suppression of increase in amino-nitrogen formation in the presence of this inhibitor was explained by the above workers as due to its suppression of the citric acid cycle, with the consequent blocking of the conversion of pyruvate to α-ketoglutarate, the latter being, according to Braunshtein, they only substrate capable of direct amination. In the present experiments, with an enzyme preparation free from transaminase and catalyzing the direct amination of pyruvate, cocaine in concentrations 5×10^{-3} M, inhibited the reaction by 80-85%. It

TABLE 1. The Effect of Various Inhibitors on Amino Acid Synthesis from Pyruvate and Ammonia (the partially purified enzyme preparation was obtained from liver mitochondria. Data represent means of 5-6 determinations)

Inhibitors	Concentration, M	NH_2-N increase, μM/mgN		Inhibition %
		without inhibitor	with inhibitor	
$CH_2BrCOOH$	10^{-2}	131	46,3	65
	$2 \cdot 10^{-3}$	131	50,1	60
	10^{-3}	131	90,1	30
Na_3AsO_3	10^{-2}	131	35,2	73
	$2 \cdot 10^{-3}$	131	58,9	55
	10^{-3}	131		
KCN	10^{-2}	129	21,0	84
	$2 \cdot 10^{-3}$	129	75,0	43
	10^{-3}	129	128,0	0
NaF	10^{-2}	105	23,0	80
	$2 \cdot 10^{-3}$	105	62,0	40
	10^{-3}	105	105	0
NH_2OH	10^{-2}	105	21	80
	$2 \cdot 10^{-3}$	105	53	50
	10^{-3}	105	68	35
Semicarbazide	10^{-2}	100	35	67
	$2 \cdot 10^{-3}$	100	50	50
	10^{-3}	100	61	40
Cocaine	$5 \cdot 10^{-3}$	174	29	83

is possible that cocaine, and also some of the other inhibitors tested, act upon the system indirectly, namely, by inhibiting the processes concerned with the liberation of hydrogen, which is essential for the reaction of reductive amination.

Recent investigations in the Institute of Pharmacology and Chemotherapy, AMN SSSR, showed that the antibiotic cycloserine specifically inhibited the glutamic-alanine transaminase. It is of interest that the glutamic-aspartic transaminase was not affected by cycloserine. According to the results of Vyshepan [7], the addition of cycloserine, in concentration 1×10^{-5} M, to the glutamic-alanine transaminase system inhibited glutamate formation by 90-100%. These results have also been confirmed by the present author. It was of great interest to determine the effect of cycloserine on the transaminase-independent synthesis of alanine from pyruvate and ammonia by the present preparation.

It will be seen from Table 2 that cycloserine inhibited alanine synthesis only in high concentrations, but was without effect in the lower concentrations tested. There is thus a difference of several orders of magnitude between the concentrations of cycloserine required to inhibit transaminase and those effective in the present system. This afforded a means for the determination of the respective contribution of the two processes to the synthesis of amino acids from keto acids in liver slices. With cycloserine present in 1×10^{-5} M concentrations, transamination could be taken to be almost completely inhibited, and therefore amino acids synthesized under such conditions may be assumed to have arisen as a result of direct amination of ketoacids.

Rat liver slices were incubated with pyruvate and ammonium carbonate in the presence of 1×10^{-3} and 2×10^{-3} M cycloserine. Apart from NH_2-N formation, glutamic-alanine transaminase activity was also determined under those conditions by following glutamate synthesis from alanine and α-ketoglutarate (added at 0,1 M). The glutamate formed was estimated according to Giri.

It will be seen from the results (Table 3) that under the experimental conditions glutamic-alanine transaminase activity was suppresed to zero level, while amino-nitrogen formation was approximately 50% of that observed in control incubations, where cycloserine was not present.

Since only glutamic-alanine transaminase, but not glutamic-aspartic transaminase, is inhibited by cycloserine, it was of interest to investigate cycloserine inhibition of amino acid synthesis in incubation mixtures containing oxaloacetate instead of pyruvate as a substrate. In mixtures containing oxaloacetate cycloserine was found to inhibit amino-N formation by about 30%. This may be taken as being due to the inhibition of the glutamic-alanine transaminase, which can also be expected to participate in amino-N formation in the presence of oxaloacetate, since the latter is readily decarboxylated during the incubation to yield pyruvate. The experiments with cycloserine showed that, with the glutamic-alanine transaminase practically inactive, the liver was still capable of synthesizing amino acids from pyruvate and ammonia to an extent of about 50% of that produced under normal conditions. These

TABLE 2. The Effect of Cycloserine on Amino Acid Synthesis from Pyruvate and Ammonia (a purified enzyme preparation, obtained from liver mitochondria, was used. The data represent mean values of five experiments)

Cycloserine concentration, M	NH_2-N formation, μM/mg N		Inhibition, %
	with inhibitor	without inhibitor	
10^{-2}	155	0	100
$2 \cdot 10^{-3}$	155	154	0
10^{-3}	155	161	0

TABLE 3. The Effect of Cycloserine on the Synthesis of Amino Acids from Pyruvate and Oxaloacetate in Liver Slices

Substrate	Cycloserine concentration, M	Control		Expt.		Inhibition, %	
		I	II	I	II	I	II
Pyruvate	$2 \cdot 10^{-3}$	120	240	55	0	54	100
	10^{-3}	120	240	55	0	54	100
Pyruvate	$2 \cdot 10^{-3}$	170	260	75	0	55	100
	10^{-3}	170	260	96	0	45	100
Oxaloacetate	$2 \cdot 10^{-3}$	120	260	90	0	25	100
	10^{-3}	120	260	75	0	40	100
Oxaloacetate	$2 \cdot 10^{-3}$	110	230	80	0	27	100

Note: I) NH_2 - N formation, μM/mg N; II) glutamate synthesis, μM/mg N.

TABLE 4. The Effect of Dialysis on the Purified Enzyme System and of FAD and PALPO Supplementation to the Dialyzed Preparation on Amino-N Formation (data represent means of five experiments)

Additions	NH_2-N formation, μM/mg N		
	nondialyzed preparation	dialyzed against phosphate buffer	dialyzed against acetate buffer
None	129	34	55
FAD, 2 μγ	–	129	125
PALPO, 20 μγ	–	34	70
Boiled extract, 1 ml	–	146	123

results, however, can only be taken as preliminary since the amounts of cycloserine available were not sufficient to carry out a sufficient number of experiments that would provide the final evidence.

The problem was therefore approached from another direction and attempts were made to investigate the effectiveness of the glutamic-alanine transaminase under the conditions where direct amino acid synthesis was inhibited. These conditions were approximated by the use of fluoride.

According to the present results, 1×10^{-2} M flouride inhibited the direct amination by 80-90% while, according to Vyshepan [8], this inhibitor was without effect on transamination.

The effect of fluoride on amino acid synthesis from pyruvate and ammonia was studied with liver slices. The following values for NH_2-N formation, (μM/mg N) were obtained in the presence (1×10^{-2} M) and absence of fluoride:

With NaF	Without NaF	Inhibition, %
135	60	56
143	70	49

These results have thus confirmed the earlier results obtained with cycloserine, namely, that the contribution of the glutamic-alanine transaminase to the total amino acid synthesis in liver slices was of the order of 50%.

The system catalyzing the direct amination of pyruvate was further examined for cofactor requirement. The rat liver enzyme preparation used was purified by electrophoresis on starch block and dialysed. Dialysis was carried out in a Zigmund star dialyzer at 4-5°, for 3 hours, against

TABLE 5. The Effect of Atebrine on the Activity of the Purified Enzyme System (Values represent the means of four experiments)

Atebrine concentration, M	NH_2-N formation, μM/mg N		Inhibition, %
	without inhibitor	with inhibitor	
$2 \cdot 10^{-2}$	150	0	100
10^{-2}	150	77	50
$2 \cdot 10^{-3}$	150	136	0

phosphate buffer, pH 7.4 and against acetate buffer, pH 5.2. The results are presented in Table 4.

Dialysis against either of the two buffers employed produced a considerable fall in the activity of the enzyme preparation; in a number of cases the activity fell to zero. The activity of the dialyzed preparation was fully restored by the addition of 1 ml of the boiled enzyme preparation. It was therefore of interest to test the effect on the dialyzed preparation of the most likely cofactors of the process under investigation. The cofactors tested were FAD and pyridoxal phosphate (PALPO).* It will be seen from the results (Table 4) that FAD completely restored the activity of the dialyzed preparation, while PALPO was without effect.

The results thus suggest that amino acid synthesis catalyzed by the enzyme preparation investigated, involves a flavoprotein enzyme. This suggestion also gained support from experiments on the effect of atebrine on the enzyme system.

Hellerman et al. [9], who studied catalysis involving FAD on the example of D-amino acid oxidase, showed that atebrine, as well as quinine and some of its derivatives, were bound by the apoenzyme and thus competed with FAD for the active sites. This inhibition was competitive, being inversely dependent on FAD concentration.

It will be seen from Table 5 that amino acid synthesis was completely inhibited in the presence of high concentrations of atebrine. With lower concentrations of atebrine the inhibition was only partial. The competitive inhibition by atebrine of amino acid synthesis thus provides further proof of the involvement of FAD as an indispensable cofactor in the enzyme system under investigation.

SUMMARY

The synthesis of amino acids directly from pyruvate and ammonia by a purified liver preparation was inhibited by respiratory and glycolytic poisons (KCN and NaF) only when the latter were present in the high concentrations (10^{-2} M). Inhibitors reacting with -SH groups (monobromoacetate, arsenite) or with the carboxylic groups (hydroxylamine, semicarbazide) also had to be added in high concentration (10^{-2} M) before appreciable inhibition of the direct pyruvate amination occurred.

Likewise cycloserine, which, in 10^{-5} M concentration, completely inhibits glutamic-alanine transaminase, inhibited the purified system for direct amination of pyruvate only at much higher concentrations (10^{-2} M). When the glutamic-alanine transaminase was completely inhibited by the addition of 1×10^{-3} M cycloserine, amino-N formation from pyruvate and ammonia fell only 50% of control level. Likewise, when the direct amination of pyruvate was suppresed by the addition of 1×10^{-2} M fluoride, which has no effect on the glutamic-alanine transaminase, the intensity on amino-N production again fell to about 50% of control values. These results indicate that the specific contribution of direct pyruvate amination to the synthesis of alanine in liver slices is of the order of about 50%.

The purified enzyme preparation lost all activity after 3-hour dialysis against phosphate buffer pH 7.4, or acetate buffer pH 5.2. The activity of the preparation was fully restored by the addition of FAD. This suggest that FAD is an obligatory cofactor for the system.

The author wishes to thank Professor S. Ya. Kaplanskii for valuable suggestions and advise in the course of the present work.

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* The two compounds were a gift from the laboratory of Prof. A. E. Braunshtein, and the authors are grateful to him and E. V. Goryachenkova for making the cofactors available.

† Original Russian pagination. See C.B. Translation.

THE CHARACTERISTICS OF NUCLEAR NUCLEOPROTEINS OF SOME TISSUES

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Translated from Biokhimiya Vol. 25, No. 1, pp. 112-117, January-February, 1960

Original article submitted July 1, 1959

In the deoxyribonucleoprotein (DNP) removed from tissues and isolated nuclei, along with histones or protamines there is a nonhistone protein. However, considering that the composition and properties of nuclear DNP vary depending on the method of preparation and purification, the question of the composition of native DNP of cell nuclei cannot be considered to be solved [1-3].

Vendrely and co-workers [4, 5] in a study of isolated nuclei and nucleoproteins found that from the ratio DNA/arginine in DNP they could judge the presence in its composition of either histone or protamine, and DNP samples which contained histone practically did not differ from each other in the DNA/arginine ratio. The authors considered DNP to be a nucleohistone or a nucleoprotamine. For each P atom in the isolated nuclei there was about 0.33 molecule of arginine, and after many reprecipitations of the DNP, about 0.25 molecule. Such a comparative study of DNP is of great interest; however, we must consider that DNP composition depends greatly on the method of isolating the nuclei and the number of reprecipitations of the preparation. As Perevoshchikova showed [6], in reprecipitation a considerable part of the DNP is lost, and first of all there is splitting off of a nonhistone protein. On the other hand, histones themselves are not homogeneous and can split into histone fractions rich in arginine and lysine [3, 7]. Considering these facts, Vendrely et al. in recent work [8] determined the content of lysine and histidine along with arginine. However, since these authors isolated nuclei with citric acid [9], the protein composition of the nuclei and the extracted DNP could be greatly changed. According to experiments carried out in our laboratory [10], the isolation of cell nuclei in concentrated solutions of sucrose which contain glycerophosphate can preserve the native state of the nuclei, and the nucleoproteins obtained from them are preserved almost completely in reprecipitation.

Since DNP from different sources can differ in content of nonhistone protein, which may be related to degree of differentiation and functional characteristics of the tissue [6, 11, 12], we determined the content of DNA and arginine in nucleoproteins extracted from cell nuclei isolated in sucrose with glycerophosphate [10] from different tissues which differed from each other in degree of differentiation or in function. Parallel determinations were made of the nucleotide composition of DNA, and in some of the DNP, also of the lysine and histidine.

MATERIALS AND METHODS

Cell nuclei were obtained from liver, spleen, and brain of rats, livers of hens, liver of 19-day-old chicken embryos and whole 11-day-old embryos, and also from the sperm of Azov-Black Sea sturgeon (Acipenser guldenstadtii colchisus, v. Marti).

In all cases the nuclei were first isolated by the two-stage sucrose-glycerophosphate method [13]. Before extraction of the DNP the globulin fraction was extracted once with 0.05 M potassium citrate, and then twice with 0.14 M NaCl. DNP was extracted with 1 M NaCl to complete removal, and was precipitated by dilution with six volumes of water. The resulting fibers of DNP were wound on a rod. Then the solution was centrifuged to collect the small, fine grained precipitate which was combined with the main material. The DNP which was obtained was washed and dried with alcohol and ether and used for analysis.

Nitrogen was determined by the micro-Kjeldahl method, phosphorus according to King [14], and DNA according to Dische [15]. A small sample of the preparation was also hydrolyzed in a sealed ampule for 3 hours at 137°, and arginine was determined in the hydrolyzate according to Dumazert and Poggi [16].

In the DNP preparations from rat spleen and brain and from sturgeon sperm part of the hydrolyzate was submitted to paper electrophoresis at 180 v for 5 hours in an acetate buffer, pH 5.8, for separation of the three groups of amino acids [17]. In the group of diamino acids and in the whole hydrolyzate we determined the total amino acid content by reaction with ninhydrin [18], on the basis of which was calculated the molar percent of diamino acids in the hydrolyzate. In the diamino acid fraction obtained in the same way we determined histidine according to MacPherson [19] and arginine by the method mentioned above [16]. In another portion of the hydrolyzate paper electrophoresis was carried out at 350 v for four hours in 0.05 N Na_2CO_3 at pH 11.5, and in this way we succeeded in isolating argine and lysine [20], which were determined in the extracted spots, colored by ninhydrin, by photometric measurement of the copper complex [21]. The results were

calculated in percent of each diamino acid in the preparation, assuming a 16% nitrogen content, and we calculated the mole ratio of all three diamino acids to one atom of phosphorus.

For determination of the nucleotide composition, 3-5 mg of the DNP preparation was hydrolyzed with 0.07-0.1 ml of 72% perchloric acid in a sealed ampule for one hour on the boiling water bath. The nitrogenous bases were separated by paper chromatography in solvent systems [22]: methanol-HCl-H_2O (70 : 20 : 10), and in the same system with saturation of the chamber by a mixture of isopropanol-HCl-H_2O which lessens the R_f of thymine so that it does not occur with the front of the solvent [23]. The spots were detected with a bateriocidal mercury-quartz lamp fitted with a UFS-1 light filter. The spots were cut out, eluted with 0.1 N HCl and the extracts were submitted to photometry with an SF-4 Spectrophotometer at two wavelengths against a control sample [24].

RESULTS AND DISCUSSION

Composition of the Deoxyribonucleoproteins

The results of the analysis of DNP preparations from isolated cell nuclei of different tissues are given in Table 1. The composition of DNP isolated from cell nuclei of the organs of rats and chickens (Table 1) was practically the same, the ratio of nitrogen to phosphorus was 4.1-4.3; of DNA to arginine 5.1-5.7; per atom of phosphorus there was 0.32-0.36 mole of arginine. Slight variation from these figures occurred in the DNP from chicken embryos, but analysis of only one preparation did not allow us to draw conclusions about the reality of this difference. DNP from sturgeon sperm differed sharply from the above preparations. The N/P ratio here was about 3, of DNA to arginine from 3.1-3.5 and per atom of P of DNA we found 0.52-0.59 molecule of arginine.

In Table 2, we give the content of diamino acids in DNP from cell nuclei of rat spleen and brain and sturgeon sperm, obtained by paper electrophoresis of the hydrolyzates and separate determination of each amino acid. We see that in content of diamino acids of the DNP of rat spleen and brain there is certainly no difference, which would exceed the limits of error of the determination. The DNP from sturgeon sperm is richer in arginine and histidine, but poorer in lysine than the previous two preparations. In all three preparations the sum of the molecules of diamino acids per atom of DNP phosphorus shows no difference and is about 0.8.

In Table 3 we give the molar content of diamino acids in the same three types of DNP determined by two methods: reaction with ninhydrin and separate determination of the three diamino acids distributed on a paper electrophoretogram.

The total content of diamino acids, calculated from the results of determining each of them, somewhat exceeds the value obtained by the reaction with ninhydrin and calculated by the average molar extinction. The molar percent of diamino acids of the total amino acid content in the hydrolyzate determined by this method does not exceed 20.

Analysis of these results shows the considerable homogeneity of the composition of DNP from nuclei of somatic cells and in this respect confirms the conclusions of Vandrely and co-workers. However, in distinction from the work of these authors, who found in isolated DNP about 0.65 mole diamino acids per atom of DNA phosphorus [8], we found a value not below 0.80. Of this 0.80 about 0.33 mole occurred in somatic DNP as arginine which corresponded to the value given by these authors rather for the nuclei which they isolated than for reprecipitated DNP, for which they found the value of about 0.25 molecule of arginine per atom of DNA phosphorus [5]. Thus, our results agree with the data of these authors as to the resemblance of DNP from different tissues in the ratio between arginine and the other amino acids to DNA and differ in this ratio in DNP containing protamine. However, the value of the molar ratio of arginine and the sum of the diamino acids to DNA phosphorus in our isolated DNP is higher than in the DNP isolated by the above authors and approaches the ratio found by them in isolated nuclei. This difference is evidently related to the fact that the procedure of isolating the nuclei with citric acid which was used by Vendrely et al. [9] could lead to loss of part of the nuclear proteins and to destruction of the native nucleoprotein complex of the cell nuclei [3, 25].

The method which we used probably assures better retention of proteins and nucleoproteins of the nuclei and gives a DNP which corresponds more closely to the native state of the complex which is present in the nuclei [10]. The ratio which we found is closer to the value obtained by Davison and Butler who found that in DNP of the calf thymus 85% of the phosphate groups were blocked by amino acids of the basic character [26].. This complex contains not only proteins with a basic character, but also nonhistone proteins, which is indirectly confirmed by our investigation. The results obtained in the study of DNP from sturgeon sperm gives a basis for thinking that this DNP also contains protein which is different from protamines or histones. This question is of special interest since the content of proteins of a nonbasic nature in the nuclei of fish spermatozoa has been denied by a number of authors [27].

Nucleotide Composition of DNP

The results of the determination of nucleotide composition of DNA obtained from DNP are given in Table 4. In all the preparations the Chargaff rule was followed. The ratio $(A + T)/(G + C)$ in the rat organs was about 1.3, that is, somewhat lower than in our previous work [28], which probably depends on differences and errors of method. For sturgeon sperm we obtained practically the same value of $(A + T)/(G + C)$ as Emmanuel and Chalkoff for the DNA of salmon sperm [29]. For organs of the chicken the ratio $(A + T)/(G + C)$ is somewhat lower, reaching

TABLE 1. Composition of DNP Extracted from Isolated Nuclei (in percent DNP, assumed to contain 16% N; N content in air dried preparation)

N	P	N/P	DNA	Arginine	DNA/arginine	Arginine (mole) per atom DNA-P
			Rat liver			
14,5	3,68	4,3	39,9	6,94	5,75	0,32
14,6	3,85	4,1	40,6	7,40	5,50	0,33
			Rat spleen			
15,1	3,81	44,2	36,4	6,84	5,32	0,34
			Rat brain			
15,4	3,87	4,1	39,0	6,96	5,60	0,33
15,2	3,85	4,1	37,7	6,85	5,50	0,33
			Chicken liver			
14,8	3,69	4,3	39,6	7,75	5,12	0,36
			11-Day-old chicken embryo			
15,0	3,70	4,3	38,8	8,21	4,73	0,39
			Liver of 19-day-old chicken embryo			
14,9	3,42	4,7	38,4	—	—	—
			Sturgeon sperm			
15,6	5,40	3,0	48,3	13,8	3,50	0,52
15,8	5,29	3,0	47,0	14,6	3,22	0,57
15,6	5,50	2,9	47,5	15,3	3,10	0,59

TABLE 2. Content of Diamino Acids in DNP Extracted from Isolated Nuclei

Source of preparation	Content in g per 100 g DNP			No. of molecules of amino acid per atom of DNA-P			
	arginine	lysine	histidine	arginine	lysine	histidine	all diamino acids
Rat spleen	7,9	7,7	0,91	0,352	0,414	0,046	0,812
Rat brain	8,1	7,5	0,73	0,383	0,425	0,039	0,847
Sturgeon sperm	13,5	4,0	1,86	0,530	0,184	0,080	0,794

DNP assumed to contain 16% N.

TABLE 3. Molar Content of Diamino Acids in DNP Extracted from Isolated Nuclei (in moles per 100 g DNP. Figures in parentheses, mole % diamino acids)

Source of preparation	Total determined with ninhydrin		Separate determination after electrophoresis			
	all amino acids	diamino acids	arginine	lysine	histidine	all amino acids
Rat spleen	0,58	0,092(16)	0,045	0,053	0,006	0,104
Rat brain	0,50	0,090(18)	0,046	0,051	0,005	0,102
Sturgeon sperm		0,110	0,078	0,027	0,012	0,117

1.10-1.17, and is almost the same for the liver of the chicken embryo and adult chicken.

The difference of the DNA of chickens and rats is slight and cannot therefore be considered to be firmly established, the more so that, according to the data in the literature, for DNA of chicken erythrocytes the ratio of (A + T)/(G + C) is about 1.3 [30].

SUMMARY

The ratio of DNA to arginine and the molar ratio of arginine and other diamino acids to DNA phosphorus in deoxyribonucleoproteins obtained from isolated cell nuclei of different organs of the rat and chicken are practically the same.

TABLE 4. Nucleotide Composition of DNP from Nuclei

Content in mole %				Purine / Pyrimidine	A + T / G + C
A	G	C	T		
Rat liver					
28,3	21,5	22,2	28,0	0,99	1,29
28,3	22,8	21,9	27,0	1,04	1,24
Rat brain					
29,0	21,6	21,8	27,6	1,02	1,30
Rat spleen					
29,4	20,6	21,5	28,5	1,01	1,37
Chicken liver					
27,3	24,4	23,3	25,0	1,07	1,10
11-day-old chicken embryo					
27,7	23,4	22,6	26,3	1,04	1,17
Sturgeon sperm					
30,3	21,1	21,5	27,1	1,05	1,35
28,7	21,8	21,4	28,1	1,02	1,32

In deoxyribonucleoproteins from sturgeon sperm there is relatively more DNA, arginine, and histidine, and less lysine than in the deoxyribonucleoproteins of rat and chicken organs. The ratio of DNA/arginine in it is lower than in the other deoxyribonucleoproteins studied.

The molar ratio of the sum of diamino acids to DNA phosphorus in all the DNP studied is about 0.8 which shows the presence of proteins in them which are not entirely of a basic character. The molar ratio of arginine to DNA phosphorus in DNP from chicken and rat organs is about 0.33. This value is evidently characteristic for the native deoxyribonucleoproteins of the nuclear complex.

The nucleotide composition of the DNA in the preparations of DNP studied is of the A-T type and corresponds to the data for the DNA of vertebrate animals. In the nucleotide composition of DNA of chicken embryos and adult chickens no difference was found.

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TYROSINE OXIDATION IN RAT LIVER SLICES

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Translated from Biokhimiya Vol. 25, No. 1, pp. 118-123, January-February, 1960

Original article submitted July 3, 1959

Previous studies in our laboratory on tyrosine oxidation in the liver [1, 2] ascertained that this process was drastically disturbed in animals with protein deficiency. It has been shown that this disturbance is related mainly to the inadequate production of α-ketoglutaric acid in the livers of rats maintained on protein-deficient diets. Transamination between tyrosine and α-ketoglutaric acid is the first step in the chain of reactions leading to total oxidation of tyrosine, and thus α-ketoglutaric acid deficiency retards tyrosine oxidation. It was also discovered that α-ketoglutaric acid deficiency develops as the result of disturbances in the citric acid cycle which are due to protein deficiency. Whereas addition of α-ketoglutaric acid to liver slices from rats with protein deficiency rapidly restored tyrosine oxidation to normal values, the addition of citric acid was without noticeable effect. From this it was concluded that when protein deficiency exists, the enzymatic transamination between tyrosine and α-ketoglutaric acid is not affected, but the retardation in tyrosine oxidation is due to reduction in α-ketoglutaric acid production. After the publication of the above experiments, publications appeared by Knox et al. [3-5] which showed that after injection of tyrosine into the animal adaptive formation of tyrosine-α-ketoglutaric transaminase occurred. It was also shown that injection of hydrocortisone together with tyrosine into adrenalectomized rats sharply increased the adaptive formation of this enzyme. Injection of hydrocortisone or corticosterone alone facilitated the adaptive formation of the enzyme, too.

In the light of the new data it was of interest to study the rate of tyrosine oxidation in the liver under different conditions, for it could be assumed that in many cases one of the factors producing decrease in tyrosine oxidation was the disturbance in formation of tyrosine α-ketoglutaric tranaminase.

It was also of interest to clarify the question: Does adaptive formation of a given enzyme take place not only in vivo, but also in liver slices in vitro? In the study of an analogous process, namely the adaptive formation of tryptophan peroxidase by Efimochkina [6], it was shown that the formation of this enzyme took place not only in the whole organism, but also in liver slices.

Velikodvorskaya [7] showed that protein-deficient rats retained the ability toward adaptive formation of tryptophan peroxidase in vivo, but the ability of liver slices to produce this enzyme in vitro was disturbed.

EXPERIMENTAL

Liver slices from rats weighing 120 to 150 g were used in these experiments. The slices were incubated and shaken for two hours at 37° in a Ringer-bicarbonate buffer (pH 7.4) to which were added tyrosine alone (10 micromoles per 200 mg of tissue), tyrosine together with α-ketoglutaric or other keto acids, and ascorbic acid at the rate of 2 mg per sample. At the end of incubation proteins were precipated with 6% trichloroacetic acid, and the nonoxidized tyrosine in the filtrate was analyzed by the method of Udenfried and Cooper [8]. Unincubated samples that were immediately treated with trichloroacetic acid served as controls.

RESULTS

Before presenting results of the present experiments, a condition should be mentioned which on one hand complicated the experiments and on the other hand helped to clarify certain properties of the processes in adaptive formation of enzymes. In the majority of experimental rats during the autumn of 1958 and winter of 1959 tyrosine oxidation by liver slices was very low, only 20-28% of available tyrosine rather than 40-45%, i.e., oxidation was twice lower than the norm.

In order to obtain normal values for tyrosine oxidation by rat liver slices α-ketoglutaric acid was added to the medium; this addition increased the rate of oxidation to normal values or higher (50-60% tyrosine oxidation). In these experiments oxidation in the slices took place in such a way (under optimal conditions) that it evidently could not reflect itself in values showing the rate of adaptive formation of tyrosine α-ketoglutaric transaminase in the in vivo and in vitro experiments.

From the values in Table 1 it can be observed that in one hour after injection, the increase in the rate of tyrosine oxidation in liver slices was relatively small; after three hours the increase was considerably more evident and was aroung 50%. After five hours the increase in oxidation rate was almost 80%. Comparison of these values with the ones of Knox et al, who observed a six- to tenfold increase in enzyme activity after five hours, shows that the rate of adaptive formation of enzyme in the present rats took

TABLE 1. Rate of Tyrosine Oxidation in Rat Liver Slices in Percent of the Added Quantity following Various Time Intervals After Intraperitoneal Injection of Tyrosine into Rats. In these and subsequent experiments rats were injected with 90 mg of tyrosine. Control rats were injected with physiological saline without tyrosine. Oxidation of tyrosine given in % of starting material

Hours after injection of tyrosine into rats					
1		3		5	
control	exptl.	control	exptl.	control	exptl.
21,7	33,0	25,9	41,9	22,8	37,9
28,5	26,6	27,6	36,8	25,9	43,4
—	35,0	25,6	53,7	20,5	52,1
—	35,4	—	35,7	31,1	34,7
—	39,1	—	41,6	22,9	48,3
—	—	—	34,5	—	47,1
—	—	—	31,5	—	39,6
—	—	—	—	—	49,3
Mean 25,1	33,8	26,3	39,4	24,6	44,1
% increase —	34,7	—	49,8	—	79,3

place at a much slower rate; this was most probably due to the previously mentioned defect. A different picture was observed when the rate of adaptive formation of the enzyme was studied in liver slices to which α-ketoglutaric acid was added.

Table 2 shows that after addition of α-ketoglutaric acid to slices, optimal conditions for tyrosine oxidation were produced, and in this case prior injections of tyrosine did not influence the rate of its oxidation. Since adaptive formation of the enzyme in these experiments should go on at the same rate as in the previous experiments, it can be assumed that the twofold increase in total enzyme activity in the presence of α-ketoglutaric acid did not influence total rate of reaction in liver slices.

In studies of the influences of ketoacids other than α-ketoglutaric on tyrosine oxidation in liver slices, it was observed that pyruvic acid in almost all cases significantly increased the rate of oxidation. Citric acids has less influence on the rate. Respective comparative results are presented in Table 3.

The influence of citric acid was undoubtedly related to its conversion in the slices into α-ketoglutaric acid. It is somewhat more difficult to explain the influence of pyruvic acid. If this influence was related to the formation of α-ketoglutaric acid in the Krebs cycle, then the rate of tyrosine oxidation in the presence of pyruvic acid should not be higher than in the presence of citric acid. However, in almost all of the experiments the rate of tyrosine oxidation in the presence of pyruvic acid was higher than in the presence of citric acid. This served as the basis for the assumption that the transamination reaction could proceed not only between tyrosine and α-ketoglutaric acid, but also between tyrosine and pyruvic acid. The results of LaDu and Greenberg [9] contradict this assumption for they have observed that in extracts of acetone-dried liver powder, pyruvic acid slowed down the process. In the work of Canaellakis and Cohen [10], which was conducted on purified dog liver transaminase, it was also observed that pyruvic acid could not be substituted for α-ketoglutaric in tyrosine transamination.

TABLE 2. Rate of Tyrosine Oxidation in Rat Liver Slices at Various Time Intervals after Tyrosine Injection. Rate of oxidation was determined after addition of α-ketoglutaric acid to the slices. Slice weight: 200 mg, amount of added α-ketoglutaric acid – 2 mg. Tyrosine oxidation given in % of starting material

Hours after injection of tyrosine into rats					
1		3		5	
control	exptl.	control	exptl.	control	exptl.
81,6	71,6	77,7	71,5	77,6	66,5
68,6	68,1	72,6	71,5	65,8	82,6
	85,6	81,7	95,9	60,8	64,3
	85,2	—	66,6	83,7	90,5
	90,5	—	91,2	90,9	82,0
	—	—	92,1	77,5	82,6
	—	—	85,2	—	94,0
Mean 75,1	80,2	77,3	80,0	76,1	80,3
% increase —	6,8%	—	3,5%	—	5,5%

TABLE 3. The Influence of Pyruvic and Citric Acids on Tyrosine Oxidation by Rat Liver Slices. Tissue weight 200 mg; pyruvic acid addition 2 mg; citric acid addition 2 mg. Tyrosine oxidation in % of starting material

Without ketoacid	With pyruvic acid	With citric acid
21,7	51,5	41,8
28,5	45,7	34,3
28,7	42,0	38,5
27,5	47,5	36,7
25,6	47,3	45,3
22,8	48,4	48,4
20,5	33,7	26,5
25,9	39,9	34,6
22,9	55,3	38,5
31,1	63,6	56,9
Mean 25,5	47,5	40,2
% increase —	86,2	57,3

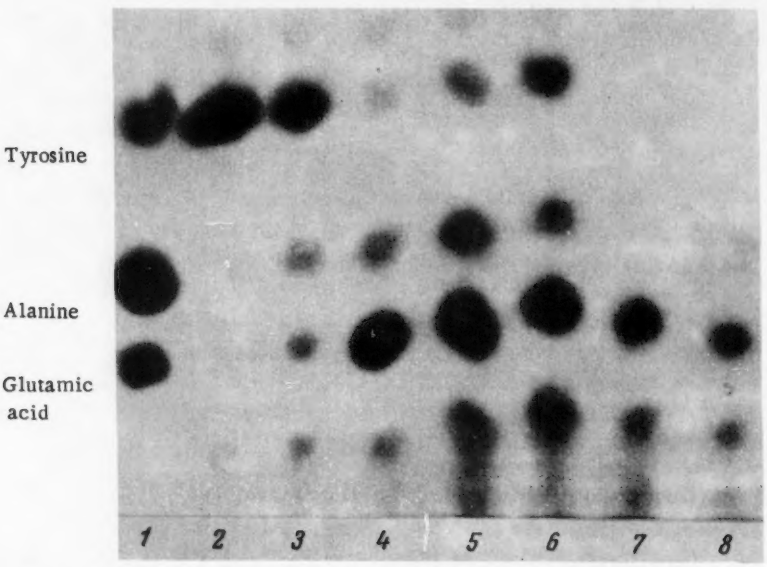
Since in liver slices addition of pyruvic acid in many cases resulted in the same degree of tyrosine oxidation as in the presence of α-ketoglutaric acid, chromatography of the reaction end products was used to clarify the question.

Samples were incubated for two hours with tyrosine and α-ketoglutaric or pyruvic acids, then they were fixed with TCA and filtered. TCA was removed from the samples with ether; the samples were then dried and redissolved in 0.2 ml of 80% alcohol containing HCl and subsequeltly chromatographed in a butanol-acetic acid-water system (4 : 1 : 5). The chromatograms were developed with ninhydrin (illustration).

In the presented chromatograms it is evident that after incubation of liver slices with tyrosine and pyruvic acid there was formation of glutamic acid and a large amount of alanine, whereas in the presence of pyruvic acid alone there was little, if any, alanine formed. Thus it can be assumed that in the slices, aside from the basic enzyme system which catalyzes transamination between tyrosine and α-ketoglutaric acid, there is a system which catalyzes transamination between tyrosine and pyruvic acid. It could also be assumed that as a result of transamination between endogenous glutamic acid and the added pyruvic acid, alanine and α-ketoglutaric acid were formed, and the latter one entered into the transamination reaction with tyrosine [11]. This last step, if it did occur, could not play a prominent role, for in the chromatograms prior to tyrosine incubation there was little if any glutamic acid observed.

The possibility of adaptive formation of tyrosine α-ketoglutaric transaminase in liver slices in vitro was studied in a series of experiments. In order to clarify this formation, liver fragments that were preliminarily incubated with tyrosine for 1-3 hours, were washed with a physiological saline and subsequent tyrosine oxidation was studied by the previously described method. The results are presented in Table 4.

Table 4 shows that in five out of nine experiments preincubation of liver slices with tyrosine had a significant influence on subsequent tyrosine oxidation. The increase in the rate of oxidation amounted to 25-79.8%. In two experiments the preincubation influence was much less noticeable and amounted to only 8-10.3%; in two



Chromatograph of amino acids in butanol—acetic acid—water system after incubation of liver slices with tyrosine and α-ketoglutaric, pyruvic, or citric acid. 1) Control; 2) tyrosine (after incubation); 3) tyrosine; 4) tyrosine + α-ketoglutaric acid; 5) tyrosine + pyruvic acid; 6) tyrosine + + citric acid; 7) α-ketoglutaric acid; 8) pyruvic acid.

TABLE 4. The Influence of Tyrosine Preincubation on Its Subsequent Oxidation. Numbers represent micromoles of oxidized tyrosine. Weight of slices 200 mg, amount of added added α-ketoglutaric acid 2 mg

No. of expts.	Control fragments	Interval of tyrosine preincubation, in hours		
		1	2	3
5	3,12	3,90	5,46	5,61
6	3,45	5,33	5,14	4,95
12	7,36	—	8,12	—
16*	5,04	5,54	5,80	5,40
17*	5,65	5,51	5,53	5,18
19	2,89	4,13	3,76	4,44
25	4,97	—	5,01	—
33	4,76	—	6,46	—
35	2,78	—	3,93	—

Amount of α-ketoglutaric acid, liter/mg

experiments preincubation was without influence on the subsequent rate of oxidation.

The causes of observed differences in the adaptive formation of tyrosine-α-ketoglutaric transaminase cannot be explained at the present time; however, increases in the rate of tyrosine oxidation were considerably higher than the possible errors of estimates; this warranted the conclusion that adaptive formation of this enzyme took place not only in the whole organism, but also in isolated liver slices in vitro.

DISCUSSION

The results presented concerning the rate of tyrosine oxidation in the liver under varying conditions warrant the conclusion that in the normal animals the rate is determined primarily by the rapidity of transamination between trosine and α-ketoglutaric acid, and also possibly by transamination between tyrosine and pyruvic acid. The speed of the above reaction depends on the rate of formation of these ketoacids in the citric acid cycle. Any disturbances of this cycle that result in lowering of α-ketoglutaric acid formation are reflected in the rate of transamination and subsequent tryosine oxidation. It is of considerable interest that the adaptive formation of tyrosine oxidation enzymes, in analogy to tryptophan oxidation enzymes, can take place not only in vivo, but also in isolated tissues in vitro. It is possible that in liver cells there are inactive components of respective enzyme systems from which, after incubation of the tissue with tyrosine, there are formed active enzyme systems.

Clarification of this question will be the subject of further experimentation.

SUMMARY

The rate of tyrosine oxidation in normal rat liver is determined primarily by the rate of formation in the citric acid cycle of α-ketoglutaric acid, and possibly also of pyruvic acid, with which tyrosine interacts by transamination. This is the first reaction in the tyrosine oxidation cycle.

The adaptive formation of tyrosine-α-ketoglutaric transaminase was observed not only after administration of tyrosine to intact rats, but also after addition of tyrosine to isolate liver fragments in vitro.

I wish to express my appreciation to Professor S. Y. Kaplanskii for supervision of this study.

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DETERMINATION OF C-TERMINAL GROUPS OF PLANT PROTEINS BY THE THIOHYDANTOIN METHOD

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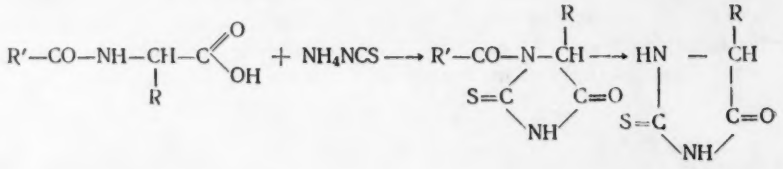
Translated from Biokhimiya Vol. 25, No. 1, pp. 124-128, January-February, 1960

Original article submitted July 4, 1959

Various methods are used for the determination of C-terminal groups in proteins, but none of them are free from some difficulties [1-6].

In the investigation of C-terminal groups of plant proteins we have used the thiohydantoin method suggested by Schlak and Kumpf [7] in 1926 and used by a number of later investigators [8-12]. This method is based on the formation of a thiohydantoin ring in the reaction of the protein with ammonium thiocyanate in a medium of acetic anhydride according to the following scheme:



The resulting thiohydantoin derivative of the protein is split and the separate thiohydantoin is identified by paper chromatography either as such [8, 9], or as a free amino acid which is obtained by splitting the thiohydantoin [10, 11, 13].

The latter process may obviously involve the formation of by-products which arise on the chromatogram and also may cause destruction of some of the amino acids by hydrogen bromide [13]. This possibility may be the reason for the fact that Baptist and Ball [13] did not regard this method as suitable to determine C-terminal groups in insulin which were exactly established by other methods. Therefore, the method of chromatography of the hydantoins seems more hopeful, and we used it in the present work.

The investigation was carried out with wheat gliadin and hemp edestin. Chief attention was devoted to the stage of washing the thiohydantoin derivative of the protein from the byproducts of the reaction which five numerous spots on the chromatogram and in making the process of splitting the thiohydantoin derivative of the protein more accurate.

The recommended process of splitting the thiohydantoin by the action of HCl [9] leads to formation of numerous side substances which give long spots on the chromatogram. The method recommended by Haurowitz [14] is more useful; it consists of heating the thiohydantoin derivative of the protein at 55° with 0.01 N alcohol solution of HCl for 150 hours. Complete splitting of the thiohydantoin was shown by a radiometric method. However, in our experiments this method led to the formation of obviously parasitic spots (in the case of gliadin).

When we used the method of Landman and co-workers [15] for splitting the thiohydantoin derivative of the protein, a method suggested for splitting phenylthiohydantoin derivatives of proteins in the determination of N-terminal groups by the Edman process, we obtained a clear spot corresponding to that of the standard without parasitic spots. In this process the splitting of the thiohydantoin from the peptide chain of the protein takes place by the action of a solution of hydrogen chloride in dioxane at room temperature for two hours. Completeness of splitting was controlled by repeated experiments described below.

MATERIALS AND METHODS

Gliadin was extracted from Erythrosperm 875 wheat by extraction of gluten with 65% ethyl alcohol [17]. The elementary composition of the gliadin was: N 15.54%, S 1.04%; according to the literature: N 17.66%, S 1.03%. Edestin was isolated from hemp by extraction with 10% NaCl solution [16].

Synthesis of 5-substituted 2-thiohydantoins from amino acids used as standards for chromatography; this was carried out by the method described in the literature [17, 18] from chemically pure amino acids.

For most amino acids we used the following method [18]: a mixture of 2 g of amino acid, 2 g ammonium thiocyanate, 9 ml of acetic anhydride and 1 ml of glacial acetic acid was heated on the water bath to solution of the precipitate, and 50 ml of water was added. The precipitate of acetylthiohydantoin was recrystallized from

water on ethyl alcohol and hydrolyzed by HCl. The resulting thiohydantoin was recrystallized from water or ethyl alcohol. To obtain the thiohydantoins from cystine and cysteine, which are not soluble in acetic acid we used the process of Nicolet [19] with preliminary acetylation of the amino acid in alkaline medium.

The thiohydantoins corresponding to glutamic and aspartic acids were synthesized by a round-about route, since these acids do not react with ammonium thiocyanate directly [20-22]. The synthesis of the thiohydantoin corresponding to glutamic acid was carried out as follows: 5 g of glutamic acid was transformed into the pyrrolidonecarboxylic acid by heating at 100-150° for 20 min, then at 150-160° for two hours [23]. The product was repeatedly extracted with boiling acetone, in which glutamic acid is not soluble. The pyrrolidone carboxylic acid was transformed into acetylthiohydantoin [24] which was heated with HCl (1 : 1) passed into the thiohydantoin; this was recrystallized from water, m. p. 120° (m. p. 122° according to [24]). The thiohydantoin corresponding to aspartic acid was obtained according to Johnson [21].

Obtaining the thiohydantoin derivatives of proteins: One g of protein was added gradually with intensive mechanical stirring to a mixture of acetic anhydride (9 ml), glacial acetic acid (1 ml), and anhydrous ammonium thiocyanate (0.55 g), heated to 100°. Heating was continued for 30 min. After cooling, the solution was poured into 100-200 ml of cold water and left for 12-20 hours. The precipitate was washed with water, dried at 50° and ground in a mortar. To remove the sulfur containing admixtures we washed the product in a Soxhlet apparatus for two days with benzene, then for one day with methanol to complete disappearance of substances which give a color with the Groth reagent used in the development of the chromatogram [25] (drop test on paper).

We showed that the side products washed out in this way gave long spots in chromatography with the following R_f values (solvent, butanol saturated with water): 0.35, 0.66, 0.8, 0.1. Solutions of thiourea give a spot with R_f 0.50. These spots can distort the results of chromatography in the study of thiohydantoins and therefore it is important to wash out carefully the byproducts formed in the synthesis.

Splitting the thiohydantoin derivatives of the proteins was carried out with a dioxane solution of hydrogen chloride [15]. The dioxane solution contained 21.8% HCl by weight. The washed thiohydantoin derivative of the protein was treated with 5 ml of the HCl solution in dioxane and kept two hours at room temperature; then, in a vacuum desiccator with alkali, it was freed from hydrogen chloride and dioxane. The solid residue was repeatedly treated in the cold with ethyl acetate or an alcohol-ether mixture (1 : 1) to remove the split thiohydantoin and the extracts were combined and evaporated to 1-2 ml. The resulting solution was chromatographed.

To test the completeness of separation of the thiohydantoin we submitted the solid residue to a supplementary saponification under more drastic conditions according to Haurowitz [14] by the action of an alcoholic HCl solution (1 ml of 1 N HCl per 100 ml of 96% ethyl alcohol) at 55-60° for 150 hours or by the action of 2 N aqueous HCl at 70° for 2-3 hours [15] with later drying of the product in a vacuum and extraction with ethyl acetate or an alcohol ether mixture (1 : 1).

Chromatographic study of the thiohydantoins. Chromatographic paper from the Leningrad paper factory was soaked in a phosphate buffer solution with pH 6.3. From a number of solvents which we studied we chose the following three: butanol saturated with water phenol saturated with water, and benzyl + ethyl alcohol (1 : 2). For determination of R_f we used about a 0.2% solution of the thiohydantoin, and chromatographed about 10-20 μg of substance. The average value of R_f was calculated from 20-30 determinations. The chromatogram was sprayed with Grote reagent [25] to show the spots. Attempts to change to the Chesley reagent [26] gave unsatisfactory results since the spots were not clear.

RESULTS AND DISCUSSION

The splitting of thiohydantoin derivatives of proteins by a dioxane solution of hydrogen chloride was most satisfactory; a clear spot was obtained on the chromatogram, individually identified by the corresponding standard. Supplementary splitting according to Haurowitz [14] gave no product which formed a spot with the Grote reagent. With supplementary splitting by 2 N HCl at 70° extended parasitic spots were formed. The appearance of the chromatograms is shown schematically in Figs. 1 and 2.

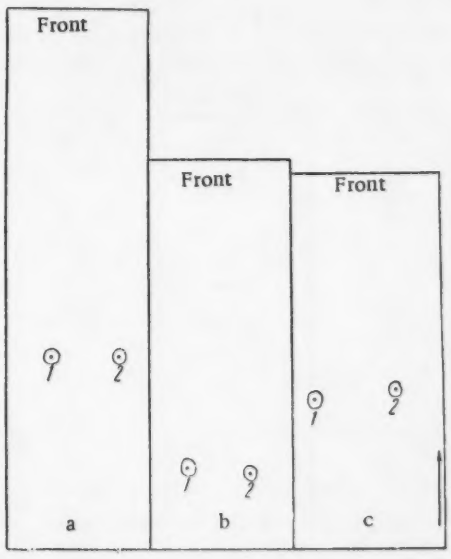


Fig. 1. Chromatogram of the thiohydantoin from gliadin (1) and from glutamic acid (2): a) Solvent, benzyl alcohol-ethyl alcohol (1 : 2); b) solvent, butanol saturated with water; c) solvent, phenol saturated with water.

Average R_f Values of the Thiohydantoins

Proteins and standards	R_f values in		
	butanol + water	phenol + water	benzyl alcohol + ethyl alcohol (1 : 2)
Gliadin	0,26	0,33	0,40
Glutamic acid	0,22	0,35	0,41
Gliadin	0,20	0,40	0,35
Glutamic acid	0,21	0,44	0,35
Edestin	0,12	0,17	0,28
Aspartic acid	0,12	0,18	0,28
Edestin	0,10	0,11	0,25
Aspartic acid	0,10	0,11	0,28

As the figures show, there is close agreement in the values of R_f of the thiohydantoins obtained from gliadin and edestin and those from glutamic and aspartic acids, respectively, in all three solvents.

In the table we give the R_f values obtained from two series of experiments which also confirm the C-terminal amino acid in gliadin is glutamic acid and in edestin, aspartic acid.

The literature contains only the work of Ramachandran [27] on the determination of the C-terminal groups of gliadin. Ramachandran identified the C-terminal group of gliadin by chromatography of the free amino acid obtained by splitting the corresponding thiohydantoin by the action of hydrogen bromide according to Baptist and Ball [13]. Ramachandran found as the C-terminal amino acids in gliadin glutamic acid and leucine. He obtained the same results using the method of reducing gliadin with lithium borohydride. These results contradicted the results of the work of Baptist and Ball who showed that glutamic acid was split by the action of HBr. Therefore, Ramachandran considered his results preliminary.

The results of our experiments agree with the data of Ramachandran on the presence of glutamic acid as a C-terminal group of gliadin, but we did not find leucine.

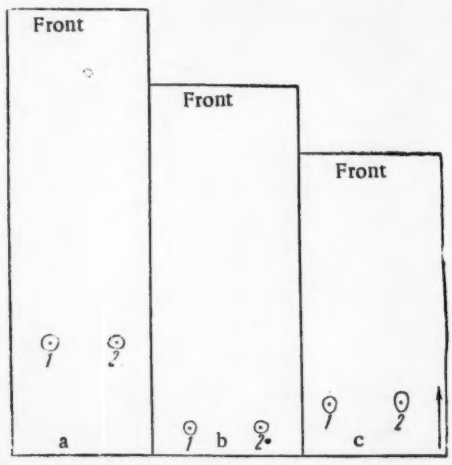


Fig. 2. Chromatogram of the thiohydantoin from edestin (1) and from aspartic acid (2): a) Solvent, benzyl alcohol-ethyl alcohol (1 : 2); b) solvent, butanol saturated with water; c) phenol saturated with water.

In the work of Haurowitz [14] it was shown quantitatively that in gliadin and edestin there are C-terminal groups which react with ammonium thiocyanate, but he did not determine the nature of the C-terminal groups. There are no results in the literature on the nature of the C-terminal groups of hemp edestin.

When we interpret the results of the work of Ramachandran and of ourserves we consider that free glutamic and aspartic acids in the interaction with ammonium thiocyanate and acetic anhydride do not form thiohydantoins, but give acid anhydrides [20-22]. However, the amides of these acids form thiohydantoins with ammonium thiocyanate. Therefore we can assume that in the terminal amino acids of gliadin and edestin there are not free carboxyl groups, but amidated carboxyl groups.

SUMMARY

We have showed the possibility of using the thiohydantoin method with later chromatography of the resulting thiohydantoins for the determination of the C-terminal groups of plant proteins, and we have made the method more accurate by washing and splitting the thiohydantoin derivatives of the plant proteins.

We have showed that the C-terminal group of wheat gliadin is glutamic acid, evidently in the form of its amide, and the C-terminal group of hemp edestin is aspartic acid, also in the form of the amide.

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DIFFERENCES IN RADIOSENSITIVITY OF DEOXYRIBONUCLEOPROTEINS AND DEOXYRIBONUCLEIC ACID

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Translated from Biokhimiya Vol. 25, No. 1, pp. 129-134, January-February, 1960

Original article submitted July 8, 1959

In connection with the great biological significance of deoxyribonicleic acid, there have been many investigations in recent years on the radiosensitivity of these compounds and the transformations which they undergo under the influence of ionizing radiation. Thus, the work of Taylor [1], Butler [2], Limperos and Mosher [3], and others has shown that in vitro under the action of an average dose of radiation (a thousand to ten thousand roentgens) there is a decrease in viscosity of solutions of these compounds which occurs spontaneously after irradiation has stopped. A considerably smaller amount of work has been devoted to the study of the radiosensitivity of the protein-nucleic complex, the nucleoprotein.

In recent years Pasynskii and co-workers [4] have showed a high radiosensitivity of nucleoproteins, greatly surpassing the sensitivity to radiation of serum proteins and lipoprotein complexes. The presence of high radiosensitivity of nucleoproteins has also been shown in the work of Kuzin [5].

However, comparative analysis of the radiosensitivity of deoxyribonucleoproteins (DNP) and deoxyribonucleic acid (DNA) has been quite insufficient, and there are very contradictory views. Thus, in one of several studies devoted to this question, Sparrow and Rosenfeld [6] compared the sensitivity to radiation of a 0.4% solution of nucleohistone and a 0.2% solution of the sodium salt of DNA. According to the results of these authors, DNP is more stable toward radiation than is DNA. Kaufman [2] obtained contradictory results with DNP gels, and also with solutions of DNA and artificial complexes of DNA with beef albumin; he found great stability of DNA to the action of radiation as compared to DNP.

In recent years it has been shown [17] that nucleoproteins differ sharply in a number of physical parameters from the DNA found in their composition, and the change in these properties is very closely connected with the amount of protein in the complex. If we consider that in cells DNA is bound to protein (and it is possible that at different stages of mitosis the amounts are different), then it is clearly important to study the effect on radiosensitivity of DNA of the amount of protein found in the natural nucleoprotein complex.

In spite of the importance and immediacy of this problem, it has has little attention in the literature. The problem of our work is to give a comparative study of the radiosensitivity of DNA and DNP with different protein contents.

METHOD

Obtaining DNA and DNP. We obtained the starting preparation of DNP from fresh frozen calf thyroid by several modifications of the method of Mirsky and Pollister [7]. The temperature, time of extraction, and number of reprecipitations corresponded to the conditions of the original method. This permitted us to obtain highly polymerized preparations with similar ratios of nitrogen to phosphorus. In a considerable number of starting preparations N/P varied within the limits 3.5-4.0 at a limiting viscosity about 20 $\frac{100 \text{ ml}}{\text{g}}$.

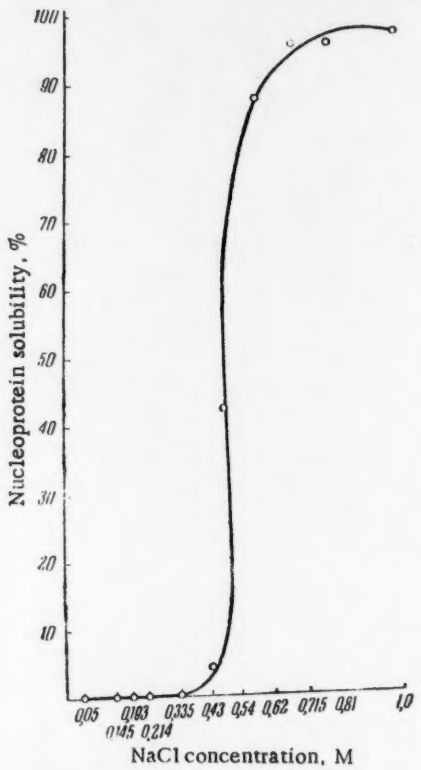

Fig. 1. Solubility of nucleoprotein (N/P about 3.6) depending on NaCl concentration in the solution.

A number of starting DNP preparations were studied for degree of homogeneity. It is known that one of the indexes of homogeneity is the solubility curve. In Fig. 1 we show the typical curve of dependence of DNP solubility on NaCl concentration, from the nature of which it follows that most of the nucleoprotein precipitates in very narrow limits of change of the salt composition of the medium. This can be one of the indications of homogeneity of the starting DNP solution.

Nucleoproteins with a lower protein content in the complex were obtained by deproteinization of the starting DNP. This procedure was carried out by the method of Sewag et al. [8] by treating the DNP solution with a mixture of chloroform and n-butyl alcohol in the ratio 3 : 1 and later separating the resulting emulsion by centrifuging. The entire operation was carried out at 2-4°. For the study of the radiosensitivity we selected preparations after the first deproteinization, with an average deproteinization close to the point of the break in solubility, and after the final deproteinization.

The majority of American and English investigators who have studied the radiosensitivity of DNA have used lyophilically dried preparations dissolved to the desired concentration. However, at the present time there are data which show that DNA is not at all insensitive to the drying operation. Therefore we kept the preparations of DNP and DNA in the cold in the form of salt solutions without preliminary drying. We studied the possibility of a protective action during irradiation of the chloroform and n-butanol which were present in the solutions of the preparations. For this purpose we added either chloroform or butanol before irradiation to solutions of the starting DNP which had not been submitted to Sewag deproteinization and which therefore could not contain interfering admixtures. Then after irradiation we compared the time of outflow, and hence the specific viscosity of the preparations which did or did not contain these substances. It is known that the limiting solubility of chloroform in water is about 1%. Since for irradiation the starting solution was diluted 35-50 times, the effect of chloroform was studied at a concentration of 0.1%. (3-5 times higher than it could be in the experiments), and no protective action was found. The limiting concentration of butanol in water is about 10%. Hence its protective action was studied at a concentration of 0.5% or less. With an irradiation of an 0.004% DNP solution with a dose of 10,000 r the threshold of protective action lies between 0.05% and 0.01% n-butanol. It follows from these data that after dilution of the DNP or DNA solutions before the work the butanol concentration will be such as to assure more or less protective action. Naturally this is very undesirable and can distort the picture of the comparative radiosensitivity of DNP and DNA. To avoid this, the starting DNP preparation was freed from butanol either by reprecipitation from 0.14 M NaCl solution or by dialysis against a salt solution. The resulting preparations of DNP and DNA were characterized by the nitrogen content (Kjeldahl-Conway method) and the phosphorus content (Fiske-Subbarow method). In some cases the nitrogen was determined by the Nessler micromethod and the phosphorus spectrophotometrically by the method of Spirin [9]. The percent content of protein and nucleic acid in the nucleoprotein complex was calculated from the N/P ratio. Viscosity determination was carried out in an Ostwald viscosimeter with a capillary diameter 0.6 mm and capillary length 100 mm at 25° ± 0.01°; we determined the characteristic viscosity. The value of the limiting viscosity was calculated from the equation of Roskin [10] which for DNA and DNP gives entirely satisfactory agreement with the method of graphic extrapolation to zero concentration.

From the date of characteristic viscosity we calculated the mol. wt., degree of a symmetry, and mean square of the distance between the ends of the molecule of the preparations ($\sqrt{R^2}$·A) [11-13]. Solutions of the starting preparations of DNA and DNP had concentrations of about 0.1-0.2% DNA. In this range of concentrations there was a great enough intermolecular interaction [14, 15]. In order to investigate the radiosensitivity at the purely molecular level before irradiation of the starting solution we diluted in to 0.003-0.004%. The source of radiation was an X-ray apparatus for close focus surface thereby RUT-60-20-1 with a short focus tube for examining light alloys, with a beryllium window, BPV-60. Irradiation was carried out with a constant distance of focus in a plexiglass container. Variation in dose was produced by changing the current strength and time of irradiation, which assured the same spectral composition of the radiation in all the experiments. Special experiments showed that with these exposures there was no heating of the irradiated solutions.

RESULTS AND DISCUSSION

Experiments in the study of the radiosensitivity of DNP and DNA were run on preparations in which the N/P ratio was varied from 3.6 to 1.7. As an example, we give the characteristics of the preparations in three series of deproteinizations (Table 1).

As Table 1 shows, all the preparations were of sufficiently high molecular weight. It is important that the DNA in them did not undergo any changes as a result of carrying out the deproteinization. We can judge this by the fact that in the DNA solutions as compared to the DNP solutions the characteristic viscosity was twice as great and the degree of asymmetry and the mean square distance were greater by about 30%. Also, in DNP solutions with a decreased amount of proteins as a result of deproteinization the viscosity practically equalled the viscosity of the starting DNP. Hence, carrying out the deproteinization did not change the degree of polymerization of the preparation.

One of the important and generally accepted tests of the native nature of DNA is ϵ(P), the coefficient of molar extinction relative to 1 mole P [16]. Our value for ϵ(P) for the DNA which we studied was quite satisfactory:

TABLE 1. Characteristics of Starting Preparations of DNP and DNA

Deproteinization series	Preparation (figures are number of deproteinizations)	N/P	Protein content in complex, %	Limiting viscosity number $[\eta]\frac{100\ ml}{g}$	Asymmetry of molecule (a/b)	Mean square of distance between ends of molecule $\sqrt{R^2}$ Å	Mol. wt. DNP	Mol. wt. DNA in the DNP
1	DNP, starting	3,6	54	19	205	2680	$9,6\cdot10^6$	$4,2\cdot10^6$
	DNP-1	3,1	46	19,8	209	2740	$8,6\cdot10^6$	$4,4\cdot10^6$
	DNP-2	2,8	41	18,5	202	2630	$7,3\cdot10^6$	$4,1\cdot10^6$
	DNP-4	2,4	31	20,0	210	2770	$6,7\cdot10^6$	$4,5\cdot10^6$
	DNP-8	2,1	22	23,9	230	3100	—	—
	DNP-22 (DNA)	1,7	5	35,8	282	4080	—	$4,0\cdot10^6$
2	DNP, starting	3,6	54	20,2	212	2780	$10,4\cdot10^6$	$4,5\cdot10^6$
	DNP-3	3,2	48	23,5	228	3090	$10,2\cdot10^6$	$5,2\cdot10^6$
	DNP-7	2,4	31	21,5	218	2900	$7,3\cdot10^6$	$4,8\cdot10^6$
	DNP-24 (DNA)	1,9	13	40,6	298	4440	—	$4,3\cdot10^6$
3	DNP, starting	3,8	59	18,1	200	2590	$9,6\cdot10^6$	$4,0\cdot10^6$
	DNP-1	3,2	48	17,0	193	2480	$7,6\cdot10^6$	$3,8\cdot10^6$
	DNP-6	2,3	29	16,5	191	2430	$5,3\cdot10^6$	$3,7\cdot10^6$
	DNP-25 (DNA)	1,8	10	31,0	261	3710	—	$3,5\cdot10^6$

Name of Preparation	Series	$\epsilon(P)_{260}$
DNA (DNP-22)	1	6650
DNA (DNP-24)	2	7180
DNA (DNP-25)	3	6400

As already indicated, irradiation was carried out in solutions with 0.003-0.004% DNA. Since the molecule of nucleoprotein contains only one DNA molecule, and, depending on N/P, different numbers of protein molecules, all the preparations compared had the same molar concentration, which is very important since the radiosensitivity, or, more accurately, the percent of damage to the substrate in the case of indirect action is to some extent a function of a molar concentration. The choice of the concentration in irradiation was connected with the fact that it assured damage at the molecular level. Most of the work devoted to a study of DNA radiosensitivity by viscosimetric methods was carried out on solutions with a concentration of 0.1-0.4%. At these concentrations there is very considerable intermolecular interaction which makes difficult the further treatment of the results.

Irradiation of the preparations was carried out at two levels, 5000 r and 25,000 r. The most obvious difference in radiosensitivity of nucleic acids and nucleoproteins was found at the lower dose, and in this case it was characteristic that the DNP solutions with N/P greater than two gave rather similar percentages of damage. In separate cases (Table 2) the starting DNP was more resistant to the action of radiation than were deproteinated preparations.

It is most important that in deproteinization the radiosensitivity increases sharply, almost in a jump, at the moment when the N/P becomes less than two. As is known, a value of N/P of about 2 is the inversion point of the change of macromolecule of DNA from the compressed state characteristic for DNP to the extended state [17]. Further decrease in N/P results in a twofold increase in viscosity and an 80% increase in the degree of asymmetry. Thus, preparations of DNA with N/P < 2 differ from DNP with N/P > 2 by a similar protein content in the complex and by the state of DNA characteristic of the extended form.

The fact that irradiation was carried out in very dilute solutions allows us to explain the damage in the macromolecules chiefly by direct action. Since in DNP the DNA molecules are surrounded by the protein envelope which can prevent access of the products of radiolysis of water to the DNA, this can be related to the greater stability of DNP to the action of X-ray irradiation. However, if we accept such an explanation, we must expect that in deproteinization, as the protein envelope is gradually decreased, an envelope which according to present ideas is not very thick, there should be a gradual increase in radiosensitivity of DNA. Our data instead shows an entirely different character of the relationship of radiosensivitity to protein content in the complex

According to the data of Butler [2] the effect of action of ionizing radiation on DNA is analogous to the action of heat. It is known that heating within definite limits results in fall in the viscosity of DNA without decrease in molecular weight, that is, to a decrease degree of asymmetry. This decrease in a/b corresponds to transformation of DNA from the extended to the compressed state. Starting from this we can assume that small doses of radiation will mostly cause compression of the DNA molecule; with increase in the dosage the compressed form begins to be degraded with irreversible decrease in viscosity and a lowered molecular weight. If this is so, then at first the compressed molecule of DNA should be more stable than the extended one. Since in DNP with N/P >2 the DNA molecule occurs in the compressed state, the resulting DNP solutions should be more radiosensitive to a change in the degree of asymmetry than DNA solutions with N/P < 2.

Direct experiments on the comparison of radiosensitivity of solutions with compressed and extended configura-

TABLE 2. Effect of X-ray Irradiation on the Molecular Parameters of DNP and DNA at Doses of 5000 r. Irradiation conditions: 60 kv, 20 ma, filter 0.5 mm Al focus distance 100 mm

No. of deproteinization series	Irradiated preparations; figure is number of deproteinizations	Decrease in limiting viscosity $\Delta[\eta]\frac{100\ ml}{g}$	Decrease in degrees of asymmetry of molecule $\Delta\ a/b$	Decrease in mean square distance between ends of molecule $\Delta\sqrt{R^2}$ Å
1	DNP, starting	2,3(12)*	13(6,3)*	230(8,5)*
	DNP-1	3,6(18)	20(9,5)	340(12,4)
	DNP-2	2,8(15)	15(7,4)	280(10,7)
	DNP-4	4,2(17)	22(10,5)	390(14,1)
	DNP-8	5,0(21)	26(11,3)	450(14,5)
	DNP-22 (DNA)	13,0(36)	57(20,2)	1070(26,2)
2	DNP, starting	1,6(9)	9(4,5)	140(4,9)
	DNP-3	3,3(14)	16(7,5)	310(10,0)
	DNP-7	3,4(16)	18(9,0)	310(10,7)
	DNP-24 (DNA)	16,2(40)	65(21,8)	1280(28,8)
3	DNP, starting	2,4(13)	14(7,5)	230(9,7)
	DNP-1	1,7(10)	9(4,9)	160(6,9)
	DNP-6	2,7(16)	16(9,1)	270(12,4)
	DNP-25 (DNA)	9,6(31)	45(20,8)	820(28,4)

* Figures in parentheses indicate decrease, in percentage.

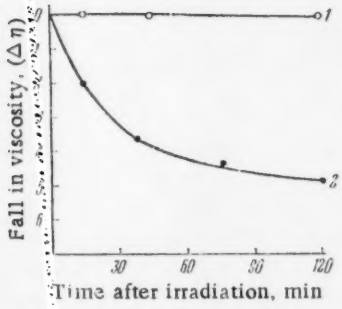

Fig. 2. Results of radiation in solutions of DNA and DNP: 1) DNP (N/P about 3.6); 2) DNA (N/P < 2).

tion of the DNA molecule show the reality of this assumption [18].

As our data showed, DNP solutions differed from DNA solutions not alone in greater radiosensitivity. One of the typical properties of DNA is the "effect of the consequences." This means that after irradiation is stopped, for a period of time (several hours) there is a fall in viscosity of the irradiated solution. Differing from DNA, solutions of the starting DNP after irradiation at a dosage of 10,000 r do not show the consequential effect (Fig. 2). The possibility is not excluded that these results are also related to the change of the extended form of DNA to the compressed; and the DNA, compressed in the nucleoprotein structure, within definite limits of dosage where there has not yet begun a definite destruction of the molecule, shows no notable effect of fall in viscosity after stopping the irradiation. This idea is favored by our data that DNA which contains less 20% protein, but has gone over into the compressed state, does not show these results as compared to the same acid under conditions of extended configuration of the molecule.

SUMMARY

We have obtained and characterized nucleoproteins with different protein contents. We have shown that when dilute solutions are irradiated by X-rays (dose 5000 r), DNP with N/P < 2 (DNA) has greater radiosensitivity than DNP with N/P > 2.

We have established that the starting DNP, as distinct from the DNA, does not show consequential effect.

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ENERGY METABOLISM OF CANCER CELLS

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Translated from Biokhimiya Vol. 25, No. 1, pp. 135-142, January-February, 1960

Original article submitted July 10, 1959

The energy metabolism of cancer cells has been extensively studied. However, up to now, there has been no single idea as to the effectiveness of mechanisms of their energetics. Some investigators [1-3] consider that the respiration of cancer cells is energetically incomplete because of its uncoupling from phosphorylation processes, and other workers find the respiratory mechanism of cancers to be energetically complete [4, 5].

Besides this, the question of the content in the cells of energy rich compounds, in the synthesis of which the energy of respiration and glycolysis is accumulated, is not simple. There is reason to suppose that in the actively growing cells the ATP level is maintained not alone because of phosphorylation coupled with respiration or glycolysis. Recently papers have appeared [6-8] concerning the fact that ATP shared in the structural components in the synthesis of nucleic acids and thus some of the substances needed for the biosynthesis of cells are constantly removed from the sphere of action of the energy metabolism. The decrease in ATP in the growing cell cannot be compensated by synthesis de novo from low molecular weight precursors.

The possibility of synthesis of adenine nucleotides from low molecular weight compounds is different when cells respire because of endogenous substrates of a fatty nature, when glycolysis occurs, or when both energy mechanisms coexist. We have already mentioned that aerobic glycolysis and respiration share in anabolic reactions not only as processes which supply cells with energy, but also as mechanisms which form a very large number of low molecular weight compounds which are included as materials in the structure of the fatty substances of cancer cells [9]. Thus, considering the energetic possibilities of cancer cells in the presence of glycolysis, respiration, and their coexistence, it would be incorrect to consider only data which characterize the intensity of their coupling with the phosphorylation processes [3].

We report below the results of experiments on the isolation of ATP from cancer cells after their incubation under conditions of anaerobic glycolysis, respiration, and the coexistence of the two energy mechanisms. After we had separated the free nucleotide chromatographically, we determined not only the amount of ATP, but also the intensity of phosphorylation by the rate of uptake of radioactive phosphorus by the ATP molecule. The synthesis of ATP de novo from low molecular weight precursors was recorded by the uptake into the molecule of carbon labeled formate and by the absolute increase in absorption of the eluate at 260 mμ.

MATERIALS AND METHODS

Cancer cells of various strains, taken from rats and mice (Ehrlich ascitic carcinoma, Yoshida sarcoma, hepatoma, sarcoma 37, sarcoma 180) were washed with physiological saline and incubated in a Warburg vessel in Ringer bicarbonate buffer which contained P^{32}. After 20-30 min incubation, the cells were centrifuged and repeatedly washed in the cold with physiological saline to remove radioactive exogenous phosphorus. Free nucleotides were extracted from the cells with trichloracetic acid and isolated chromatographically by the method worked out by Venkstern and Baev[10] for the separation of free nucleotides from erythrocytes.

To 0.4 ml of cancer cells we added 0.8 ml of a solution of 7.5% trichloroacetic acid and extracted the nucleotides in the cold for 10 min. The trichloroacetic acid extract was deposited on chromatograph paper in the amount of 0.1 ml per spot (for each sample we used 0.4 ml). The spots were deposited in the middle of the paper strip at intervals of 2.5 cm. For separation of the intracellular mineral phosphorus in the ascending direction we passed an acid solvent: n-butanol, n-propanol-85% formic acid (10 : 40 : 30) for six hours (nucleotides did not migrate in this solvent, but remained at the start). The paper with the mineral phosphorus was cut out and sprayed with the reagent of Hanes and Isherwood [11] in the modification of Burrows and co-workers [12] to show the places on which it was distributed. The phosphorus was eluted from the paper with 0.01 N HCl and determined quantitatively according to Lowry et al.[13]. Separation of the nucleotides was carried out in the descending direction using an alkaline solvent: n-propanol-NH_4OH (25%)-water (60 : 30 : 10). The solution was passed twice for 48 hours. The nucleotide spots, after viewing in the ultrachemiscope were cut out and eluted with 0.01 N HCl. The nucleotide content was determined in the spectrophotometer by absorption in the ultraviolet at 260 mμ. The amount of ATP was determined not only by the amount of absorption

* The present work was carried out with the participation of N. A. Veresot-skaya.

of the eluate in ultraviolet, but also by the content of total phosphorus. The ratio of phosphorus and adenosine in the ATP eluate corresponded to the theoretical value. We measured the radioactivity of the ATP eluate and the mineral intracellular phosphorus. The intensity of phosphorylation was estimated by the value of the relative specific activity (RSA-ratio of specific activity of ATP phosphorus to specific activity of intracellular mineral phosphorus times 100).

In the experiments with labeled C^{14}-formate we first used acid solvents for separation of organic acids and amino acids. Separation of the nucleotides was carred out as described above in alkaline solvent. The uptake of C^{14}-formate in ATP was determined with an end counter after drying the corresponding eluate and was calculated per 40 μg of substance.

RESULTS

Without adding glucose to the medium the cancer cells showed steady (as to time), intense utilization of oxygen because of endogenous substrates of a fat-like nature [14]. Under these conditions we studied the phosphorylation coupled with the respiratory mechanism. Glycolytic phosphorylation was studied by adding glucose to the medium and switching respiration. Finally, under conditions approaching those physiological for the cell, that is, with aerobiosis and the presence of glucose in the medium, phosphorylation of the cancer cells occurred because of two energy mechanisms – respiration and glycolysis.

The comparison of the value of the RSA of ATP phosphorus (Table 1, samples 1 and 3) showed that respiration and glycolysis of cancer cells were practically equal in respect to intensity of the coupling of phosphorylation with them. In only some of the experiments (87, 60, 50) was the glycolytic mechanism somewhat more effective than respiration. With a single passage of glycolysis and respiration the value of the RSA of ATP phosphorus did not increase (Table 1, sample 2). The absence of increase in

TABLE 1. Respiration, Glycolysis, and Phosphorylation in Ascitic Cancer Cells (calculated per 1 ml wet cells. Incubation: 30 min)

Expt. No.	Cancer	Composition of sample	Respiration, μl O_2	Glycolysis in mg lactic acid	P-ATP	
					μg	RSA
46	Ehrlich carcinoma	Zero sample			67	
		Cells (aerobic)	462		64	68
		Cells + glucose (aerobic)	145	8,10	89	77
		Cells + glucose (KCN)		12,72	58	62
59	» »	Zero sample			91	
		Cells (aerobic)	750		83	50
		Cells + glucose (aerobic)	330	6,06	116	60
		Cells + glucose (KCN)		11,46	97	65
60	» »	Zero sample			91	
		Cells (aerobic)	480		81	50
		Cells + glucose (aerobic)	225	5,64	107	54
		Cells + glucose (KCN)		9,24	97	67
87	» »	Zero sample			56	
		Cells (aerobic)	450		53	42
		Cells + glucose (aerobic)	240	5,88	74	65
		Cells + glucose (KCN)		9,60	63	61
81	Yoshida sarcoma	Zero sample				
		Cells (aerobic)	735		104	54
		Cells + glucose (aerobic)	300	4,32	134	64
		Cells + glucose (KCN)		8,88	122	63
83	Sarcoma 37	Zero sample			81	
		Cells (aerobic)	675		64	59
		Cells + glucose (KCN)		10,44	84	52
82	Hepatoma	Zero sample			109	
		Cells (aerobic)	945		100	50
		Cells + glucose (aerobic)	675	7,56	131	64
		Cells + glucose (KCN)		12,90	126	57
85	»	Zero sample			67	
		Cells (aerobic)	735		71	57
		Cells + glucose (aerobic)	465	6,18	89	54
		Cells + glucose (KCN)		11,58	77	64
88	»	Zero sample			43	
		Cells (aerobic)	900		42	50
		Cells + glucose (aerobic)	540	6,48	66	50
		Cells + glucose (KCN)		11,82	56	50
96	»	Zero sample			73	
		Cells (aerobic)	1020		92	53
		Cells + glucose (aerobic)	645	7,08	113	63
		Cells + glucose (KCN)		13,92	99	53

RSA of ATP phosphorus in the presence of the two energy mechanisms is explained by the reciprocal inhibition of glycolysis and respiration observed for various cells of the Ehrlich ascitic carcinoma even in 1951 [4] and characteristic for other strains of malignant tumors (Table 1).

The amount of formation of lactic acid and the intensity of utilization of oxygen in the presence of the two energy processes is always less than in conditions in which the cancer cells exist due to glycolysis or respiration alone (Table 1, experiment No. 88).

In this experiment the direct Pasteur reaction occurred to the extent of 45% while the inverse Pasteur reaction, consisting in the suppressing of respiration by the process of glycolysis, was 40%. Thus, inhibition of each process by the other was about the same. If we consider that the intensity of phosphorylation due to glycolysis and to respiration was the same, then we can understand that the value of the RSA of ATP phosphorus under conditions when both processes occur at the same time does not increase, but remain unchanged. The ATP content in the cells in respiration due to endogenous substrates, on glycolytic phosphorylation, and on coexistence of the two energy mechanisms remains strictly constant. It is possible that the content of this mononucleotide, which accepts macroenergetic phosphate, limiting the rate of phosphorus turnover in the cell, assures a definite coordination of aerobic and anaerobic metabolism and the intensity of phosphorylation.

Besides this, the content of ATP in the cell as we have already showed is determined not only by the intensity of the process of dephosphorylation and rephosphorylation. Even with an energy equilibrium of glycolytic and respiratory mechanisms, the conditions for maintaining a definite ATP level in the cell can be varied. Under conditions of anaerobic glycolysis, as a result of the transketolase-transaldolase reaction there may be intense formation of ribose, an important component for the synthesis of mononucleotides. In the presence of glycolysis and respiration still another path is added to this mechanism of pentose formation, by direct decarboxylation of glucose-6-phosphate. Finally, in the last case, large amounts of three and four carbon atom compounds are formed which can also be used for plastic materials in the synthesis of mononucleotides.

Experiments on the uptake of C^{14}-formate by the ATP molecule confirmed the correctness of this idea (Table 2). Entrance of formate into the ATP molecule under conditions in which the cells respired because of endogenous substrates was many times less than in the presence of glucose in the medium, when the cells existed because of glycolysis or when respiration and glycolysis occurred together. It is interesting to observe that the radioactivity of ATP in the last two cases (except in experiment 121) was about the same. Evidently, formation of pentoses, which participate in the synthesis of mononucleotide molecules, occurs chiefly in reaction of the transaldolase-transketolase type. Uptake of formate in the ATP molecule in samples where the cells respire due to substances of a noncarbohydrate nature is explained by the presence of some quantity of endogenous pentoses. Under these conditions the synthesis of ATP de novo occurs very slightly.

The intensity of uptake of formate in the ATP molecule characterizes the synthesis of this compound de novo from low molecular weight precursors through inosinic acid, amination, and later adenylate kinase reaction. However, another path is possible in the cells for the formation of adenosine phosphoric acid from adenine and phosphoribosyl pyrophosphate [15-17] which cannot be recorded by radioactive formate.

Even in our first experiments which studied the processes of phosphorylation we observed that the amount of ATP in samples in which glycolysis occurred in the cancer cells increased during incubation (Table 1). We therefore studied especially the content of ATP after incubation of the cancer cells under different conditions by a direct method, determining their amount by the absorption of the eluate at 260 mμ after chromatographic separation of the nucleotides on paper (Table 3). The ATP content in the samples incubated without glucose compared to its amount at the moment of taking the cell from the organism (zero sample) either remained unchanged or fell slightly, on the average 8%. In samples where the cell only underwent glycolysis, there was a slight, but

TABLE 2. Uptake of C^{14}-Formate in ATP of Ascitic Cancer Cells (specific activity in counts/min · 40 μg ATP)

Experiment number	Cancer	Sample		
		glycolysis	glycolysis and respiration	respiration
70	Ehrlich carcinoma	70	81	9
71	Ehrlich carcinoma	241	192	32
104	Ehrlich carcinoma	109	100	46
68	Sarcoma 180	136	149	8
72	Sarcoma 180	120	116	19
73	Sarcoma 180	122	100	5
120	Hepatoma	124	123	40
121	Hepatoma	155	258	76

TABLE 3. ATP Content in Ascitic Cancer Cells (calculated in μg/ml wet cells. Incubation: 30 min. Figures in parentheses are change in content in percent of zero sample)

Expt. No.	Cancer	Sample			
		zero	glycolysis	glycolysis and respiration	respiration
46	Ehrlich carcinoma	439	404 (−9)	612 (+39)	439 (0)
59	» »	626	668 (+6)	792 (+20)	572 (−9)
60	» »	626	668 (+6)	732 (+18)	561 (−10)
71	» »	657	698 (+6)	850 (+29)	557 (−5)
87	» »	385	433 (+12)	510 (+32)	364 (−5)
86	» »	510	572 (+12)	566 (+11)	424 (−17)
76	Hepatoma	754	840 (+11)	874 (+15)	784 (+4)
80	»	504	575 (+11)	625 (+24)	506 (0)
82	»	750	867 (+15)	905 (+20)	686 (−8)
88	»	297	387 (+30)	458 (+54)	293 (0)
91	»	489	489 (0)	530 (+ 8)	354 (−28)
121	»	447	530 (+10)	644 (+44)	503 (+12)

quite evident, rise in ATP content, on the average 10%, after 30 min incubation. In aerobiosis and the presence in the medium of glucose, when the cells respired and underwent glycolysis, there was even a considerable increase in the amount of ATP, averaging 25%. We did not find a decrease other adenosine phosphoric acids. The ATP content remained strictly on one level, and the amount of AMP in the cells was always slight.

Assuming that the increase in absorption of the eluate from the ATP spots can be explained by the formation in the cells of IMP which in the alkaline solvents used by us has the same R_f as ATP [10], we rechromatographed the spots of ATP by the process described by Engel'gardt, Baev, and Venkstern [18]. After transfer of the ATP spots to the rechromatogram, we passed the solvent: ethanol-butanol-1 M ammonium acetate buffer, pH 3.7 (40 : 10 : 20) with simultaneous application of a standard. We did not succeed in obtaining separation of the ATP spot, which favors the idea of its homogeneity. The ratio of phosphorus and adenosine in the eluate of ATP also agrees with the theoretical value.

Thus we can conclude that the increase in the absorption of the eluate obtained from the ATP spots is related to an increase in the amount of ATP during incubation of the cancer cells.

DISCUSSION

Even a number of years ago the literature contained a discussion of the question of injury to respiration in cancer cells. It was assumed that in cancer cells there was damage to the connection of respiration with phosphorylation so that aerobic blycolysis appeared which compensated for the nergy lack in respiration. Injury to the Pasteur effect during the uncoupling of oxidative phosphorylation by the agents, it would seem, confirmed this point of view. The most positive position in this question was taken by Ach and Shtraub [2] who considered that the ATP level of cancer cells was maintained only by glycolysis; respiration, in their opinion, was energetically ineffective. The facts which we have obtained do not agree with this idea. The intensity of respirative phosphorylation in cancer cells does not yield in size to the phosphorylation connected with glycolysis. Respiration of cancer cells is energetically of full value. Under conditions where both processes occur together (aerobiosis, presence of glucose in the medium) the intensity of phosphorylation does not increase, as would be expected if we considered aerobic glycolysis as a compensatory mechanism.

A characteristic peculiarity of the metabolism of cancer cells is not damage to energetic respiration, but the appearance of an energetically full glycolytic mechanism. The outward appearance of energetically full glycolysis and respiration in cancer cells is the clear expression of mutual inhibition, that is, the simultaneous coexistence of the inverse and direct Pastuer effects. The presence of a two way relation between glycolysis and respiration determines the constant level of macroenergetic compounds in the cell, which do not increase in the presence of coexistence of two energetic mechanisms and change little in switching from one to the other. In this connection, the calculations of Warburg [3] in determining the amount of energy formed by cancerous and normal cells do not seem correct to us. These calculations are based on simple arithmetical addition of the ATP 'P which arises in the cancer cell in anaerobic glycolysis and in respiration due to endogenous substrates. Actually, such addition does not occur, since the potential possibilities of each energetic mechanism are not realized.

Our data on the energetic equality of the respiratory and glycolytic mechanisms of cancer cells, and also on the absence of increased intensity of phosphorylation in the simultaneous coexistence of the two energetic processes has been confirmed only by the work of Quastel and Bikis [19]. Estimation of the energetic effectiveness of glycolysis and respiration of cancer cells was made by these authors not by a direct method of study of the phosphorylation process, but indirectly, in a study of some reactions of biosynthesis which used for their energy macroergic compounds. This work showed that uptake of C^{14}-

glycine in the proteins of cancer cells was the same in the presence of anaerobic glycolysis, endogenous respiration, and in the simultaneous existence of the two energetic mechanisms.

Besides this, our investigations showed that the content of ATP in the cells assured not only an intensive process of dephosphorylation and rephosphorylation which occurred jointly with the reaction of energy metabolism. In spite of the energetic equality of the respiratory mechanism of cancer cells with the glycolytic, the first is unable to maintain the ATP level completely in the cells and after 30 min incubation the amount of ATP in the cells begins to decrease, though slightly. The anaerobic mechanism not only maintains a constant ATP level, but results in some increase in this compound in the cell. The optimum conditions for synthesis of ATP in cancer cells are simultaneous occurrence of glycolysis and respiration. With unchanged intensity of phosphorylation processes the increase of this compound, so important for biosynthesis in cells, is, under these conditions, about 25%. Increase of ATP in cancer cells under conditions or aerobic glycolysis was also observed by Ach and Shtraub [2]. The authors, using a microbiological method for determining ATP, showed that its content after 30 min incubation of the cancer cells increased by 30%. However, the method used by these authors did not permit accurate treatment of questions of the mechanism of this effect.

The fall in ATP observed in our experiments in cells in the presence of endogenous respiration showed that except for the continuous utilization of macroergic phosphates, there is, in the processes of biosynthesis and rephosphorylation of ADP by the reactions of energy metabolism, an expenditure of this compound in the cancer cell, compensated only by the synthesis of all the ATP molecules de novo from low molecular weight precursors. When we study the uptake of C^{14}-formate in the ATP molecule we can be convinced that the synthesis of this compound de novo actually occurs intensively under the conditions of formation of ribose in the cell, a necessary component of the mononucleotides. Under conditions of endogenous respiration, when the substrate of oxidation is a substance of noncarbohydrate character and there is no formation of ribose, the synthesis of ATP de novo is extremely limited, and its level in the cell begins to fall, since its expenditure is not compensated for by new formation.

We also have indicated that the uptake of formate in the ATP structure records only the path of formation of this mononucleotide through IMP, amination, and later adenylate kinase reaction. In the cells there is a possibility of another path of formation of adenosine phosphoric acid from adenosine and phosphoribosyl pyrophosphate, discovered by Kornberg and co-workers [15-17]. In experiments with direct determination of the amount of ATP in the cells, active increase in this compound was ground only in conditions when respiration and glycolysis occurred in the cells. This fact once again shows that the energy process not only supplies the cells with the needed energy for biosynthesis, but gives plastic materials for the synthesis of complex high molecular weight compounds.

Decrease in the ATP level under conditions in which its synthesis from low molecular weight compounds is limited permits us to believe that this compound not only shares in synthetic reactions as a donor of macroergic phosphate, but, as a simultaneous component of higher molecular weight compounds, the polynucleotides, is continuously used up in their formation. Although the share of ATP in the synthesis of polynucleotides is certain, the magnitude of this process for the growing cell is still not determined. Energetically, the multiplication and growth of cancer cells, as we have seen, has an active mechanism for ATP synthesis from low molecular weight precursors, which permits not only maintanance of a constant level of this compound, needed for biosynthetic processes, but also supplies its loss after activity.

SUMMARY

By chromatographic separation of free nucleotides from ascitic cancer cells and study of the uptake of P^{32} in ATP we have showed that intensive phosphorylation has the same value under conditions of anaerobic glycolysis as in respiration due to endogenous substrates.

Under conditions of aerobic glycolysis and respiration the intensity of phosphorylation does not increase, due to mutual inhibition of one energetic process by the other.

The synthesis of ATP in cancer cells from low molecular weight precursors through inosinic acid, indicated by the uptake of C^{14}-formate, occurs actively only in the presence of glucose in the medium, when there is formation of the important component for synthesis, pentose. Uptake of C^{14}-formate in the ATP molecule occurs with the same intensity as under conditions of anaerobic glycolysis and in the coexistence of glycolysis and respiration, which suggests the predominance of the transaldolase-transketolase mechanism of formation of pentose in cancer cells. Under conditions of endogenous respiration the uptake of C^{14}-formate into ATP is very slight.

The synthesis of ATP de novo, observed by the increase in absorption of the eluate of 260 mμ during incubation of the cancer cells, occurs actively in the presence of the two energy mechanisms, glycolysis and respiration.

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THE ISOLATION OF CELL NUCLEI BY A PHENOL METHOD AND A STUDY OF NUCLEI CHARACTERISTICS

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Translated from Biokhimiya Vol. 25, No. 1, pp. 143-150, January-February, 1960

Original article submitted July 14, 1959

It has been shown in the work of one of us [1] that when a tissue homogenate is treated with phenol, pH 6.0, after centrifuging, the DNA which enters the composition of deoxyribonucleoprotein (DNP) is concentrated in the layer at the boundary between the water and the phenol phase. The greater part of the proteins which are not bound to DNA by covalent or salt forming bonds is dissolved in the phenol. Later treatment of the DNP gel with phenol, pH 7.3,* led to liberation of native highly polymerized DNA.

When we examined microscopically the film at the boundary of the phenol and water phases after treatment with phenol, pH 6.0, we found the form of the cell nuclei was retained when we stained with methyl green, and we worked out a method for their purification ("phenol nuclei"). Since the phenol treatment fixed the tissues, suppressing enzyme activity which affected the nucleoproteins when the latter were isolated in a water medium, it seemed of interest to compare the composition of the phenol nuclei with the composition of nucleoproteins isolated from nuclei obtained in a water medium [2, 3]. Evidently, besides DNP the phenol nuclei contained other components of the nucleolus-chromatin complex, ribonucleoproteins (RNP) [4-6]. The RNA of this complex could be separated into fractions which had different rates of metabolism.

The present communication describes the isolation, cytology, and chemical composition of the phenol nuclei, and also the results of fractionation and metabolism of the RNA of the phenol nuclei.

METHOD

Isolation of Phenol Nuclei

Phenol nuclei were isolated from liver, kidney, spleen and brain of rats, calf thymus, and Ehrlich ascitic carcinoma of mice.

The tissue was homogenized in 10-20 volumes of cooled 0.14 M NaCl by 8-15 vertical strokes in the comparatively weak Potter homogenizer. The mild homogenization did not damage the connective tissue fibers, so that they were almost completely removed in the later filtration through 6-8 layers of gauze. In the work with the Ehrlich ascitic tumor instead of homogenization and filtration, the cells after removal of the ascitic liquid by centrifuging were suspended in 0.14 M NaCl. A 5-10% suspension of tissue or cells in 0.14 M NaCl was shaken for 25 min with an equal volume of phenol, pH 6.0 (0.4 ml KOH per 100 ml of phenol saturated with water) [1], centrifuged from 25 min at 3000 rpm, and the intermediate layer at the boundary of the upper water and lower phenol phases was collected. This intermediate layer consisted of two sharply bounded layers: the upper was white and rich in nuclei, the lower contained chiefly denatured cytoplasmic protein which did not dissolve in phenol.

The intermediate layer was enegertically shaken with about half its volume of a mixture of phenol, pH 6.0, and 0.14 M NaCl (1 : 1) and centrifuged 30-60 sec at 2000 rpm. As a result, the phenol and water layers separated but the intermediate film did not have time to form fully. A considerable portion of the nuclei was suspended in the water phase while the cytoplasmic admixtures were found chiefly in the phenol and partly in the intermediate layers. The water layer was collected, the phenol was discarded, and the intermediate layer was submitted to repeated shaking with phenol, pH 6.0, and 0.14 M NaCl and short centrifuging (3-4 times in all) for the fullest possible removal of the nuclei. Here the time of centrifuging was shortened still more (to 10-15 sec). The combined water suspensions of the nuclei were centrifuged 15 min at 3000 rpm, the precipitate was collected and its purity was controlled microscopically. If the nuclei were still contaminated, the above described procedure for differential purification by centrifuging was repeated once more.

The purified nuclei were shaken repeated (three times) with phenol, pH 6.0, and 0.14 M NaCl to remove all the protein and RNA which could be washed out, then centrifuged after each shaking for 5 min at 6-8000 rpm, and the intermediate layer was quantitatively collected, as pure nuclei (Fig. 1a). The phenol nuclei gave good suspensions and did not agglutinate. After the last washing the nuclei were transferred to 40% alcohol, washed with

* As further tests showed, the figures for the pH of the phenol were too low because of a defect in the potentiometer. The true pH value of the phenol used for isolation of DNA was 8.3-8.4 (addition of 2 ml of 5 N KOH/ 100 ml phenol).

70 and 96% alcohol and ether, and dried for analysis in a vacuum desiccator over $CaCl_2$. The cytology of the phenol nuclei was studied on smears taken at different stages in the isolation and stained with pyroninmethyl green [7].

In the experiments on the rate of metabolism of RNA we also carried out the isolation of "high polymer" and "low polymer" RNA [1]. For this purpose we precipitated RNA from the water phase after the first centrifuging with alcohol, dissolved it in water and precipitated the high polymer RNA with 1.5 M NaCl. Its purification from low polymer RNA was carried out by reprecipitation with 1.5 M NaCl. The low polymer RNA was precipitated with 5% TCA after removal of the high polymer form.

In a number of experiments the phenol treatment was applied to nuclei which had first been isolated from rat liver by the two-stage sucrose-glycerophosphate method [8] or from calf thymus according to Allfrey et al. [9]. After three treatments with phenol, pH 6.0, and 0.14 M NaCl, the nuclei were dried and analyzed. Part of the isolated nuclei were fractionated (extraction of the globulin fraction and the deoxyribonucleoproteins) according to the method previously described [5]. The methods of fractionating the RNA of the phenol nuclei are given below.

Analytical Procedures

The N content was determined by the micro-Kjeldahl method; P according to King [10]; nucleic acids by P and the color reaction for pentoses [11, 12] after separation of DNA and RNA according to Schmidt and Thannhauser [13].

For a study of the groups of diamino-, dicarboxy-, and monoaminomonocarboxyamino acids and especially of the molar ratio of diamino acids to DNA phosphorus we used the following method of analysis. A sample of nuclei was hydrolyzed 4 hours with 6 N HCl at 137° in a sealed ampule, the hydrolyzate was dried in a vacuum over $CaCl_2$-KOH and dissolved in a small amount of water. For determination of P a sample was taken in a micropipet for combustion, and, with the same micropipet, hydrolyzate was placed on an electrophoretogram, calculating at 0.5-1.0 mg of nuclear material per strip. Electrophoresis was carried out in 0.1 M acetate buffer, pH 5.8, at 200 v, 2.5 ma, on a strip of rapid chromatographic paper 7 cm wide and 38 cm long for 5 hours

As a result the amino mixture was divided into groups of diamino-, dicarboxy-, and monoaminomonocarboxyamino acids. The spot of the diamino acids was usually separated into two, corresponding to arginine + lysine and histidine. Control strips were colored with ninhydrin, and the portions from the others were cut out at the site of the spots. The amino acids were eluted with twice distilled water and their molar content was determined with reduced ninhydrin according to Yemm and Cocking [14]. From the resulting figures we calculated the molar ratio between the three groups of amino acids, and also the molar ratio of diamino acids to DNA phosphorus.

We also carried out determination of arginine and lysine. For this purpose the hydrolyzate was separated by paper electrophoresis (rapid chromatographic paper) in 0.05 M Na_2CO_3 at 350 v, 7 ma, on the same strips for 5 hours [15]. This separated arginine and lysine. The paper strips were dried, passed through a 10% solution of acetic acid in acetone, and the amount of arginine and lysine was determined with unreduced ninhydrin using the formation of the copper complex [16].

Determination of Uptake of P^{32} in RNA Fractions

For the study of the relative rate of metabolism in different fractions of RNA, the cells of the Ehrlich ascitic tumor were incubated one hour at 37° in the ascitic liquid to which P^{32} was added in the form of $Na_2HP^{32}O_4$ calculated at 10 microcurie per 1 ml. After incubation the cells were collected by centrifuging, suspended in 0.14 M NaCl, and by the use of the phenol treatment we isolated high polymer RNA, low polymer RNA† and phenol nuclei. The latter, as the present work showed, contained two RNA fractions, designated RNA-E and RNA-N. Fraction RNA-E was isolated from the phenol nuclei by one of the methods described below. The nucleic acid of the nuclear residue, which contained RNA-N, was extracted with 10% NaCl at 100°. Each of our preparations was hydrolyzed with 0.5 N KOH after washing, and the nucleotides were isolated by the method of Mandel and Revel [17] which consists in adsorption of the nucleotides on activated charcoal, "Norite," in an acid medium and later elution by alcohol-ammonia solution. The solution was deposited on a target, dried, and the radioactivity determined with and end counter, and then the mixture of nucleotides was dissolved in 0.5 N $HClO_4$ and the amount determined in the spectrophotometer.

EXPERIMENTAL RESULTS AND DISCUSSION

Chemical Composition of Phenol Nuclei

Using the above described methods we succeeded in obtaining nuclei from practically all mammalian tissues except muscle. In Table 1 we give the chemical composition of nuclei isolated from different tissues and characterized by a different degree of development of the nucleolar apparatus.

On the basis of DNA content the tissues studied are divided into two groups. In the case of calf thymus and rat kidney and spleen the DNA content is about 35-36% of the dry weight, which approaches the amount in DNP obtained by salt extraction [2]. In the nuclei of rat liver and brain and mouse Ehrlich carcinoma, the amount of DNA is lower (28-29%). These figures are 6-8% less than those previously obtained in the corresponding nucleopro-

† High polymer and low polymer RNA were the two fractions of RNA produced from cytoplasm and nuclear fluid which passed into the water phase when the cells were treated with phenol, pH 6.0, and 0.14 M NaCl.

TABLE 1. Chemical Composition of Phenol Nuclei

Tissue	No. of expts.	DNA, %	RNA, %	RNA/DNA × 100
Calf thymus	6	35,5*	0,72	2,03
Rat kidney	1	36,0	0,32	0,89
Rat spleen	1	35,0	0,79	2,26
Rat liver	2	28,1	2,33	8,30
Rat brain	1	28,3	3,30	11,70
Mouse Ehrlich tumor	3	28,3	4,22	14,90

* Mean error for calf thymus DNA is ±0.38.

teins [2, 8]. This difference shows the presence in the phenol nuclei of these tissues of 15-18% protein which is not in the composition of the nucleoprotein complex isolated by salt solution. Liver, brain, and Ehrlich carcinoma are characterized by strong development of the nucleolar apparatus. It seems probable that just because of the presence of nucleolus material, which has an acid protein [4-6, 18], in the phenol nuclei, the percent DNA in them is lower.

A greater difference is observed in determination of the RNA content, and especially of the RNA/DNA ratio, in the phenol nuclei of different tissues. Here there is a clear correlation between degree of development of the nucleolar apparatus and the value of the RNA/DNA ratio (Ehrlich tumor > brain > liver > spleen and thymus > kidney) which can also indicate the content in the phenol nuclei, in addition to chromatin DNP, also of RNP of nucleoli and chromosomes.

We also considered it expedient to determine the content of some groups of amino acids in the phenol nuclei, which come preferably from DNP. The ratio of number of moles of diamino acid to number of moles of DNA phosphorus is especially interesting in connection with the connection with the data of Allfrey and Mirsky [19] on the significance of polyanions and polycations in the function of cell nuclei.

This ratio for calf thymus and rat spleen on the average equals 0.8 ± 0.016 which approaches the figure obtained by Zbarskii and Ermolaeva [2] and in the laboratory of Butler [3] in isolated nucleoproteins. The molar ratio of diamino acid/monoaminomonocarboxylic acid‡/dicarboxylic amino acids for these tissues is 22 : 60 : 18. The molar ratio of lysine to arginine averages 1.7. These results show that the nucleoproteins of the phenol nuclei isolated from tissues with a weakly developed nucleolar apparatus approach in composition to the nucleoproteins isolated by salt extraction, taking account of the necessary precautions against enzymatic depolymerization.

Thus, evidently, all the materials in the composition of DNP isolated by salt extraction are found in the composition of nucleoproteins of the phenol nuclei. These results can serve as indirect evidence of the native state of the DNP complex isolated by the salt method [2, 5, 6, 8] since the electrovalent bonds of the nucleoproteins resist deproteinization by phenol (at pH 6.0) and proteins not bound to DNA are removed [1].

Study of the chemical composition of phenol nuclei shows the presence along with DNP also ribonucleoproteins, and comparison of the RNA content with the development of the nucleolar apparatus in the corresponding tissues indicates that this RNP is the material of the nucleoli.

Cytology of the Phenol Nuclei

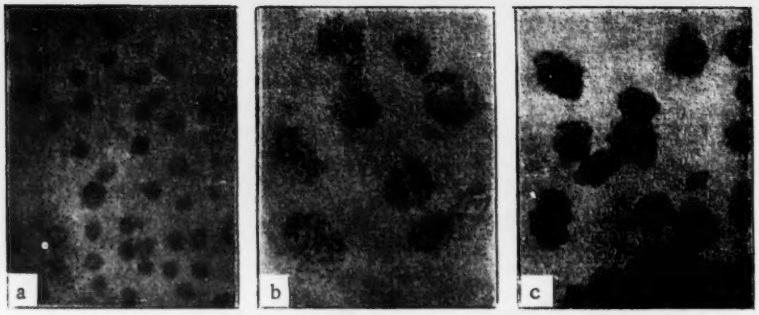
In order to explain the nature of the RNP of the phenol nuclei we studied the cytology of the latter in smears stained with pyronin-methyl green.

In the early stages of isolation of the phenol nuclei (after the first treatment with phenol, pH 6.0, and 0.14 M NaCl) the chromatin nuclei are granular and fibrous, probably corresponding to condensed chromosomes (Fig. 1b). In the latter stages of purification of the phenol nuclei they contain numerous vacuoles with walls of DNP (Fig. 1c). Some vacuoles have a larger diameter, evidently corresponding to nucleoli. In nuclei which contain much RNA, especially in the nuclei of the Ehrlich tumor, along with the DNA of the chromatin there is also RNA. Here also there is a definite relationship of distribution on stage of treatment. In nuclei after the first centrifuging a considerable part of the nucleoli contain granular structures. Sometimes it is also possible to see pyroninophilic chromosome residues. In the later stages of treatment, the pyroninophilic granules are usually fewer, but the vacuoles, especially the vacuoles which correspond to nucleoli, are stained by pyronine homogeneously or preferably toward the periphery.

From the results of the cytological analysis we can conclude that RNA of the phenol nuclei corresponds to RNA of the nucleoli and residual chromosomes, that is, RNA which occurs in the composition of the fraction of acid protein of cell nuclei [4-6, 18]. However, it remains uncertain which part of the nucleolar RNA is retained in the phenol nuclei.

For a solution of this question we isolated nuclei from rat liver [8] and calf thymus [9], suspended them in 0.14 M NaCl and shook with phenol, pH 6.0. After three to four treatments with phenol, pH 6.0, and 0.14 M NaCl, we determined the content in the nuclei of DNA and RNA. Along with this, portions of the nuclei were extracted with 0.14 M NaCl three times to remove the globulin fraction.

‡ Monoaminomonocarboxylic acids were determined by the ninhydrin method.



Phenol nuclei (stained in all cases with pyronin-methyl green): a) Isolated from calf thymus (magnified 650 ×): b) isolated from Ehrlich ascitic tumor, early stage of treatment (magnified 1350 ×); c) isolated from Ehrlich ascitic tumor, late stage of treatment (magnified 1350 ×).

Such a procedure removed the RNA of nuclear fluid, residual RNA of nucleoli, and remaining chromosomes [4-6, 18]. The globulin fraction extracted from the nuclei was also given the phenol treatment. These experiments gave the following results:

1) Practically all the RNA of the isolated globulin fraction after shaking with phenol was deproteinized and entered the water layer;

2) The ratio of DNA / protein in the nuclei after removal of the globulin fraction and after phenol treatment was the same (from this we can conclude that in both cases the same amount of protein was removed from the nuclei);

3) The amount of RNA in the nuclei treated with phenol was somewhat less (by 20-30%) than in nuclei after removal of the globulin fraction, that is, phenol treatment apparently removed besides the RNA of the globulin fraction also some part of the RNA of the acid protein.

Thus, our experiments showed that RNA of the phenol nuclei is not a chance contaminant, but is all, or in greater part, a definite part of the nuclei (nucleoli or residual chromosomes). The failure of this RNA to be extracted in the phenol treatment may depend on the specific character of this RNA complex, or the type of bond on the RNA with protein, or the structural features, for example, of its interrelations with DNA and DNP. Thus, for example, if, as is postulated by a number of authors and ourselves [5, 18, 20] it is true that there is a structural complex of nucleoli-chromatin, then it may be that the DNP of the chromosomes and the chromatin around the nucleoli under the influence of 0.14 M NaCl and later phenol treatment is condensed and blocks the outlow into the solution of the protein of the nucleoli and the residual chromosomes. However, partial solution of the latter occurs, and this can assure later vacuolization of the chromosomes and swelling of the nucleoli.

However this may be, determination of the ratio of RNA/ DNA in the phenol nuclei can characterize the development of the nucleolar apparatus in the tissues, since the RNA of the phenol nuclei is nucleolar RNA and the amount of DNA in the nucleus (chromosomes) in different tissues of one organism is the same [21]. This is the more important because the phenol nuclei can be easily obtained from the various tissues themselves (Table 1).

Fractionation of RNA of the Phenol Nuclei

As previous investigation showed [1], the results of phenol treatment under various conditions depend most of all on the type of protein in the nucleoprotein complex and the character of its bond with the nucleic acid. Therefore, evidently, phenol fractionation permits us to divide the nucleic acids which occur in different types of nucleoprotein complex, and the various metabolic functions in the cell.

We have observed that the RNA of phenol nuclei can be further fractionated by one of the processes described below.

1. Treatment in the system phenol, pH 6.0, – water or any aqueous solution with low ionic strength. If we carry out extraction of the isolated nuclei from rat liver successively with mixtures of phenol, pH 6.0–0.25 M sucrose, and phenol, pH 6.0,—water, then the resulting nuclei retain only 1/ 3 the RNA which remains after treatment with phenol, pH 6.0,–0.14 M NaCl. The same thing occurs with treatment by phenol, pH 6.0,–water with isolated nuclei of thymus gland.

In extraction of the phenol nuclei of Ehrlich carcinoma with phenol, pH 6.0,-distilled water the ratio of RNA/ DNA in them is also three times lower, though the nuclei are less pure, than after the usual treatment. Thus, independent of whether the starting material for phenol treatment comes from isolated nuclei or from whole cells, decrease in the ionic strength of the aqueous phase leads to removal of a considerable part of the RNA of the phenol nuclei (about 2/ 3). Finally, this fraction of RNA can be isolated if we first extract the phenol nuclei from Ehrlich carcinoma in the system phenol, pH 0.6,–0.14 M NaCl, and then treat their resulting nuclei with phenol, pH 6.0,-water.

2. Extraction of the phenol nuclei with phenol, pH 8.3-8.4, and 0.14 M NaCl. Treatment with phenol, pH 8.3-8.4, and 0.14 M NaCl was used to remove DNA. It

TABLE 2. Uptake of P^{32} in Different RNA Fractions from the Ehrlich Carcinoma (specific activity of RNA in counts/min · μγ RNA)

RNA fraction	Experiment 2	Experiment 3
Low polymer RNA	9.05	6.0
High polymer RNA	1.17	1.92
RNA-E of the phenol nuclei*	20.5	22.1
RNA-N of the phenol nuclei*	26.5	71.5

*In experiment 2 fractionation of the RNA of the phenol nuclei was carried out by treatment with phenol, pH 8.3 8.4, and 0.14 M NaCl; in experiment 3, with phenol, pH 6.0, and water.

was shown that with this procedure the phenol nuclei lose about 2/3 of their RNA, while 1/3 of the RNA remains in the precipitate (intermediate layer). We carried out a double extraction in which 90% of all the DNA passed into the water phase. Thus, alkaline phenol can also be used for fractionation of the RNA of phenol nuclei. It is interesting to see that even when almost all the DNA is deproteinized, one of the RNA fractions is still all retained in the composition of the nucleoprotein complex.

3. Extraction with phenol, pH 6.0, and 0.14 M NaCl of partly purified nucleolar material. The globulin fraction and DNP were removed from rat liver nuclei, so that only nucleoli, residual chromosomes (acid protein) and nuclear membranes (residual protein) remained in the precipitate. Treatment of this material with phenol, pH 6.0, and 0.14 M NaCl liberated 55% of the nucleolar and residual chromosome RNA; 45% of the RNA was not removed.

In all cases the RNA of the phenol nuclei or the RNA of nucleoli and residual chromosomes could be divided into two fractions: the extractable fraction, which we called RNA-E, and the nonextractable fraction, which we called RNA-N. We assume that RNA-N occurs in the composition of RNP and differs in properties from the other RNA of the cell. The amount of RNA-E in the phenol nuclei isolated in the sytem phenol, pH 6.0-0.14 M NaCl perhaps depends on structural factors which form the type of membrane from condensed DNP around the nucleoli and residual chromosomes. In this case, both removal of DNP (procedures 2 and 3) or its swelling (procedure 1) will make the outlow of RNA-E from the nuclei easier.

We should emphasize that our fractions RNA-E and RNA-N are not identical with the RNA_1 and RNA_2 described by a number of authors [22, 23] but rather are two subfractions of RNA_2 which is the RNA of the nucleoli and residual chromosomes [5, 6, 18].

Uptake of P^{32} in RNA of Phenol Nuclei

Since fractionation of phenol nuclei evidently reflects the existence of different types of nucleoprotein complexes in the nuclei, it is very interesting to determine the rate of uptake of labeled precursors in the two RNA fractions of the phenol nuclei and to compare this with the rate of uptake in the RNA of cytoplasm (high and low polymers).

Table 2 shows that the greatest P^{32} uptake occurs in the RNA-N fraction of nuclear RNA, the one which does not dissolve in the treatment with phenol, pH 6.0, and distilled water. The uptake in this fraction is many times higher than the uptake in high polymer RNA and several times higher than in the RNA-E of the phenol nuclei. On the basis of the fact that both methods of fractionation give the same results, we may believe that in this way we get identical fractions. The uptake in the low polymer RNA is higher than is the uptake in high polymer RNA, but it is lower than in both fractions of the phenol nuclei.

The data which we have presented shows the considerable difference in rate of metabolism of different RNA fractions obtained by the phenol method. It is possible that this is related to the fact that the RNA of one fraction is a precursor of the RNA of the other fraction. We may also believe that the RNA-N fraction, which has a higher level of uptake, corresponds to the RNA synthesized on the DNA matrix. To test this assumption we are now carrying out a study of the uptake of P^{32} in the RNA fractions with time and of the nucleotide composition of the RNA fractions of the phenol nuclei.

In conclusions we should observe that, besides the fraction of the nucleic acids of the phenol nuclei, the latter can be used when it is necessary to study the components which undergo enzymatic splitting in the isolation of nuclei in the usual media.

SUMMARY

We have described a method for obtaining pure nuclei (nucleoprotein complexes of the nuclei) by treatment of tissue homogenates with phenol, pH 6.0, in combination with later differential centrifuging. The phenol nuclei contain DNA, RNA, and protein. The percent content of DNA in the phenol nuclei of calf thymus, rat spleen and rat kidney is close to the values found in nucleoproteins isolated from nuclei by salt extraction; in the phenol nuclei of rat liver and brain and the mouse Ehrlich carcinoma, it is somewhat lower and corresponds to the DNA content of the nucleolar-chromatin complex. The amount of RNA in the phenol nuclei from different tissues varies greatly, and we have noted a strong correlation between its content and the development of the nucleolar apparatus. The molar ratio of diamino acids to DNA phosphorus in the phenol nuclei of calf thymus and rat spleen is about 0.8; the molar ratio of lysine to arginine is 1.6-1.7. We have described a simple process for determining the molar ratio of diamino acids to DNA phosphorus using paper electrophoresis.

The cytology of the chromatin of phenol nuclei in early stages of treatment shows a granular structure, and in later stages, vacuolization. The RNA of phenol nuclei is evidently the RNA of nucleoli and residual chromosomes.

This conclusion is confirmed by experiments with phenol treatment of isolated nuclei.

We have described a procedure for separating the RNA of phenol nuclei into two fractions, using treatment in the system phenol, pH 8.3-8.4,-0.14 M NaCl or phenol, pH 6.0, -water. In RNA-E, which enters the water phase, we find under these conditions 2/3 of the RNA of the phenol nuclei; in the RNA-N, which remains in the insoluble precipitate, there is about 1/3. Uptake of P^{32} in the RNA-N of the phenol nuclei in Ehrlich carcinoma cells, incubated in vitro in ascitic fluid, surpasses considerably the uptake in high polymer cytoplasmic RNA (35-80 fold), and surpasses several times the uptake in the RNA-E of the phenol nuclei. The low polymer RNA renews phosphorus considerably faster than the high polymer, but more weakly than both fractions of phenol nuclei RNA.

We have suggested the use of phenol nuclei in studying the cytochemistry of cell nuclei.

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SPECTROPHOTOMETRIC DETERMINATION OF NUCLEIC ACIDS

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Translated from Biokhimiya Vol. 25, No. 1, pp. 151-159, January-February, 1960

Original article submitted July 23, 1959

The methods in use for the determination of nucleic acids (NA) of tissues depend on the determination of one of the components of RNA or DNA - phosphorus, pentose, or nitrogenous base. Two sources of error are inherent in all the currently used methods: these are incomplete separation of RNA from DNA, and the presence in the extracts of interfering contaminants.

Schmidt and Thannhauser's method [1] gives the fullest separation of RNA from DNA, but the fractions obtained contain, apart from NA, considerable amounts of contaminants which interfere with the accurate determination of NA, whether from the P or the pentose content [2-5]. Although the content of contaminants in the extracts obtained by the procedure of Ogur and Rosen [6] is lower, it does not assure complete separation of RNA from DNA [7-9]. The application of spectrophotometric methods to the determination of DNA and RNA after their separation by the Schmidt-Thannhauser procedure would be both convenient and reliable, were it not for the presence of contaminants, chiefly protein degradation products, whose absorption in the UV cannot be ignored [5, 10, 11].

It thus appears that none of the currently applied procedures for the fractionation of NA affords the possibility of obtaining their components – P, pentose, or nitrogenous base – free of contaminants which interfere with their determination.

Various methods have been applied by a number of authors to the elimination of the effects of these contaminants, such as: shortening the time, and lowering the temperature, of alkaline hydrolysis [11], purification of RNA nucleotides by absorption of contaminants on ion-exchange columns [5] or by paper electrophoresis [2], preliminary fractionation of NA, with their preparation in a purer form [12], and specific precipitation of DNA [13]. The first and the last of these procedures do not, however, assure complete elimination of contaminants, while the remaining ones are excessively laborious.

Vierordt's method for the determination of a substance in its mixtures with other substances, depending on the difference in absorption at two different wavelengths [14-16], has been applied to the cytochemical determination of NA. This method might be applicable to the biochemical determination of RNA and DNA in the impure extracts obtained by the Schmidt-Thannhauser procedure. It has, however, been used only for the determination of total NA content [17].

The results presented in this paper demonstrate the possibility of determining the contents of RNA and DNA in the presence of interfering contaminants, by measuring absorption at two different wavelengths.

METHODS

The animals were killed by decapitation, and the portions of tissue were weighed on a torsion balance, and then homogenized in a glass homogenizer with 10% trichloroacetic acid, with cooling. After eliminating acid-soluble substances by extracting twice with cold 5% trichloroacetic acid liquid, P was removed by treatment with 96% ethanol, followed by boiling with 3 : 1 ethanol–ether mixture for 3 min, and with 1 : 1 methanol-ether mixture for 30 min, and finally by ether extraction. The residue was dried at 37°.

The dry residue was incubated with N KOH for 18 hr at 37°, taking 1 ml of alkali per 100 mg of wet weight of tissue. To the cooled hydrolyzate we added 0.1 ml of concentrated $HClO_4$ per ml of KOH solution added. The precipitate was separated on the centrifuge, and washed with two portions of cold N $HClO_4$ solution. The supernate plus washings, containing the ribonucleotides, was made up to a known volume with N $HClO_4$. DNA was removed from the precipitate by two extractions with N $HClO_4$ (30 min at 80°). The control solution for RNA determinations was N KOH, and for DNA determinations N $HClO_4$.

NA content was determined in rat liver (age about 9 months), in mouse skin and muscle (age about 3 months), and in Guerin's rat and Ehrlich mouse carcinoma tissues.

Absorption in the UV was measured in a Zeiss Universal spectrophotometer. Phosphorus was determined by digesting the material with H_2SO_4–HNO_3 mixture, and reducing ammonium phosphomolybdate with stannous chloride [18]. Ribose was determined by Mejbaum's method [19], heating for 45 min [20], and deoxyribose by Dische's diphenylamine method [21], heating for 10 min. The measurements were made on a Pulfrich photometer, using a S_{66} filter for ribose, and a S_{61} filter for deoxyribose. The results were calculated as mg P per 100 mg of fresh tissue.

When ribose was determined by the orcinol reaction, we calculated the phosphorus content from the formula

P = (E + 0.045)*/ 0.1250, and for the diphenylamine reaction with deoxyribose from the formula P = E/ 0.0195. RNA-P was derived from the UV spectrophotometer readings taking $\epsilon(P) = 10,130$; for DNA-P $\epsilon(P) = 8782$.

We prepared an alkaline hydrolyzate of the proteins of the given tissues, in order to find the most suitable wavelengths for measuring the RNA and DNA contents in the presence of non-nucleotide contaminants arising in the course of alkaline hydrolysis. With this object, we extracted the NA from the tissue powder prepared as above, by six 15-min extractions with N $HClO_4$ at 80°. The completeness of extraction was checked spectrophotometrically. The NA-free residue was hydrolyzed with N KOH, by heating on a boiling water bath for 1 hr. The alkaline hydrolyzate was treated with concentrated $HClO_4$, and diluted to a known volume with N $HClO_4$, in the same way as for the NA-containing hydrolyzates given by the Schmidt-Thannhauser procedure. The absorption curves given by the protein hydrolyzates were measured in the UV region.

RESULTS

As is evident from the table, the RNA and DNA contents of the tissues, as derived from analyses of their various components, vary widely. For all the tissues examined, the highest values for RNA content were given when these were based on UV absorption at 260 mμ, and on the orcinol reaction for ribose. Although the values obtained by these two methods agree closely with each other, and with those reported in the literature [24, 25], they must be regarded as being much higher than the real figures, owing to the presence of contaminants in the extracts. The presence of impurities which interfere with the orcinol reaction for pentoses has been demostrated [4, 5, 10]. The presence of admixtures which absorb in the UV is shown by the absorption curves of our RNA and DNA fractions, which differ significantly from those found with preparations of pure RNA and DNA (Figs. 1 and 2). The basic difference is that the NA fractions absorb very strongly at wavelengths lower than 240 mμ, and exhibit heightened absorption at 270-290 mμ. In this respect, our findings are in conformity with those of the Belgian workers [5].

These results show convincingly that determination of NA content from the UV absorption at the absorption maximum gives too high results. The close agreement between the values found for NA contents using the UV absorption and the pentose methods cannot, therefore, be regarded as being evidence of the reliability of the results. This is also shown by the data on the P content of the RNA fractions (see table), which, although lower than those derived from the UV absorption or from pentose estimations, are nevertheless too high, as follows from the establishment of the presence of nonnucleotide P in the RNA fractions obtained by the Schmidt-Thannhauser procedure [2, 3].

It is necessary, for the application of Vierordt's method for the exclusion of the effects of contaminants on the UV spectrophotometry of nucleic acids, to have the absorption spectra of RNA, DNA, and contaminants, and to show that the UV absorption curves of the NA - contaminants mixtures are the resultants of the arithmetical summation

* In agreement with the findings of other authors, we also found that the curve relating extinction found for the orcinol reaction with pure RNA to its phosphorus content intersected the abscissa axis to the right of the zero point of the system of coordinates.

NA Content Expressed as mg% Phosphorus Derived from Determination of their Components

Organ	Number of animals	RNA				DNA				RNA/DNA			
		ΔE	E_{260}	ribose	P	ΔE	E_{260}	desoxy-ribose	P	ΔE	E	pentose	P
Liver (rat)	5	54,4 ±1,3	98,3 ±2,5	99,1 ±4,9	80,0 ±3,3	29,4 ±1,6	38,1 ±1,7	—	18,5 ±0,6	1,86	2,58	—	4,32
	in %	100	181	182	147	100	131	—	63				
	5	51,6 ±1,5	92,2 ±2,1	87,4 ±2,1	74,1 ±1,9	22,0 ±1,0	30,5 ±1,8	20,6 ±1,3	14,0 ±2,0	2,37	3,02	4,24	5,29
	in %	100	179	169	143	100	139	94	64				
Skin (mouse)	5	19,2 ±1,5	39,2 ±1,8	38,4 ±2,5	—	27,1 ±2,4	38,5 ±3,4	27,5 ±1,9	—	0,71	1,02	1,40	—
	in %	100	204	200	—	100	142	101	—				
	5	17,1 ±1,3	34,4 ±2,1	33,8 ±2,2	21,8 ±1,5	24,1 ±1,3	31,1 ±1,4	26,0 ±1,5	22,7 ±1,7	0,71	1,10	1,03	0,96
	in %	100	201	188	127	100	129	108	94	1,61	1,71	—	—
Muscle (mouse)	3	4,5	23,8	—	—	2,8	13,9	—	—				
	in %	100	529	--	—	100	496	—	—				
Guerin's carcinoma	1	51,9	82,7	90,6	63,6	88,2	108,5	91,7	80,3	0,59	0,76	1,14	0,79
	in %	100	159	175	123	100	123	104	91				

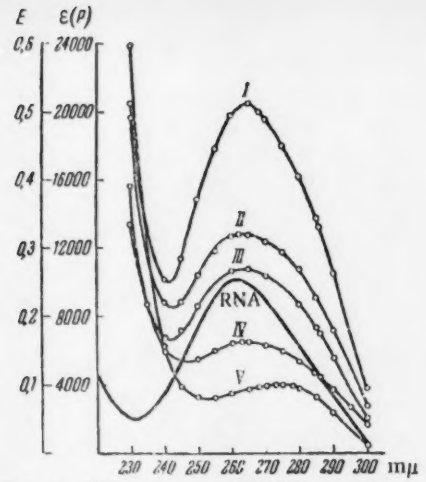

Fig. 1. UV spectra of RNA fractions prepared according to Schmidt and Thannhauser, and of pure yeast RNA. Solutions of N $HClO_4$. Ordinates axis: optical density, taking 100 mg of fresh tissue per 100 ml of extract; for RNA taking 1 g-atom P/liter. I) Ehrlich carcinoma; II) rat liver; III) Guerin's carcinoma; IV) mouse skin; V) mouse muscle.

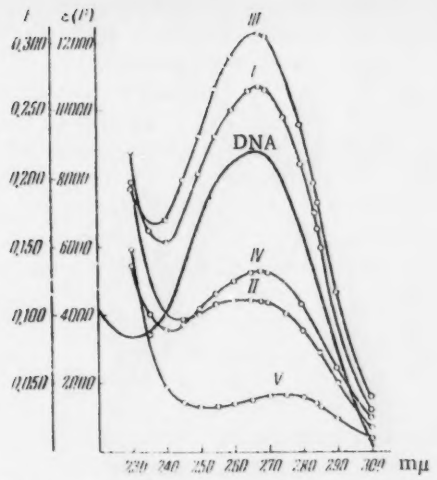

Fig. 2. UV spectra of DNA fractions extracted with hot N $HClO_4$, and of pure DNA in N $HClO_4$. Ordinate axis: optical density, taking 100 mg of fresh tissue per 100 ml of extract, and for DNA taking 1 g-atom P per liter. Numbering of curves as in Fig. 1.

of the absorptions given by each component taken separately:

$$E_\lambda = K_\lambda^N \cdot N + K_\lambda^X \cdot X, \qquad (I)$$

where E_λ is the optical density of a NA - contaminant mixture at wavelength λ, N is the content of NA, with an absorption coefficient fo k_λ^N, and X is the content of contaminant, with an absorption coefficient of k_λ^X. It follows from equation (I) that the difference between the optical density of a solution containing RNA and contaminant, at 260 mμ and at some other wavelength λ would be expressed by:

$$E_{260} - E_\lambda = N \cdot (K_{260}^N - K_\lambda^N) + X \cdot (K_{260}^X - K_\lambda^X). \qquad (II)$$

For the exclusion of the effects of contaminants it is necessary to select a wavelength λ_0, at which the absorption coefficient of the contaminants would be equal to that at 260 mμ. The second term of equation (II) would then be equal to zero, and the effect of contaminants would vanish. Then

$$N = \frac{E_{260} - E_{\lambda_0}}{K_{260}^N - K_{\lambda_0}^N} = K \cdot \Delta E. \qquad (III)$$

The concentration of NA may conveniently be expressed as P mg-%, and equation (III) then takes the form, for RNA:

$$RNA = K \frac{(E_{260} - E_{\lambda_0}) \cdot V}{w \cdot l}. \qquad (III')$$

where V is the volume of extract, $\underline{w}$ is the weight of material taken, and l is the depth of the layer of solution. The coefficient K is derived from the formula:

$$K = \frac{M \cdot r}{\varepsilon(P) \cdot (r - 1)}, \qquad (IV)$$

where M is the mol. wt. of P, $\epsilon(P)$ is the molar extinction coefficient of RNA at 260 mμ, related to a mole of P[26], and $\underline{r}$ is the ratio of the optical densities of pure RNA at 260 mμ and at wavelength λ_0. The same formula is applicable to the calculation of the DNA content, reading the optical densities at 268 mμ, and making the appropriate changes in the values of the coefficients.

The chief difficulty encountered in the selection of the second wavelength is that of finding a suitable protein standard. The use of proteins other than those of the tissues would be inadmissible. For this reason we used the proteins of the tissue under analysis for NA as our standard material.

UV absorption spectra of hydrolyzates of the proteins of the tissues under examination are shown in Fig. 3. The shape of the curves is the same for tissues of different origins (mouse skin and muscle, rat liver, rabbit gastric mucous membrane, solid Guerin's carcinoma, Ehrlich ascites carcinoma), and they differ only in the concentration of absorbing substances; the curves exhibit a maximum at 278 mμ, and a minimum at 259 mμ, and equal absorptions are found at 260 and 286 mμ, and at 268 and 286 mμ. It appears from these results that the substances liberated as a result of alkaline hydrolysis of the proteins of different tissues exhibit practically identical optical properties, irrespective of their origins.

It follows that measurment of optical density of extracts at 260 and 286 mμ, for RNA, and at 268 and 284 mμ for DNA, suffices for the elimination of the effects of contaminants on the spectrophotometric determination of nucleic acids, on the condition of compliance with the addition rule (I). In view of reported deviations from this

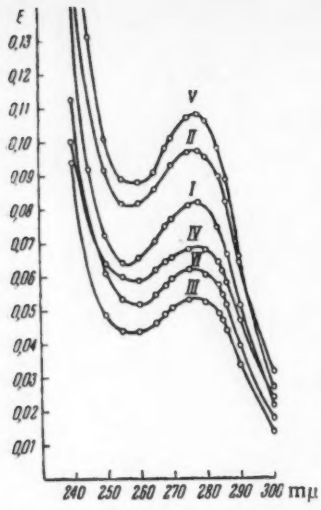

Fig. 3. UV spectra of alkaline hydrolyzates of proteins. Ordinate axis: optical density, taking 100 mg of fresh tissue per 100 ml. Numeration of curves as in Fig. 1. VI) Rabbit gastric mucous membrane.

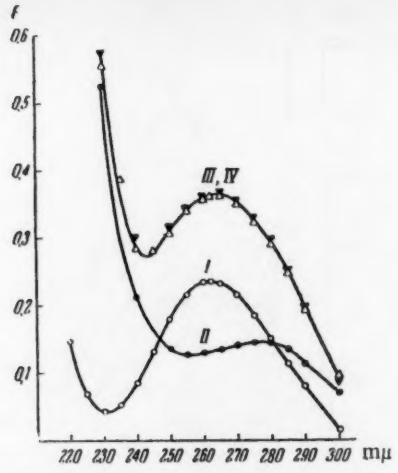

Fig. 4. Additivity of absorption in mixtures of RNA nucleotides and protein hydrolyzates. I) Absorption spectrum of alkaline hydrolyzate of pure yeast RNA; II) absorption spectrum of alkaline hydrolyzate of liver protein; III) absorption spectrum of a mixture of I and II; IV) curve given by summation of the values of I and II.

rule [27], according to which the absorption of adenylic acid – tyrosine mixtures was much smaller than was the sum of the absorptions of the components taken separately, we had grounds for doubt as to whether equation (I) was applicable to our extracts, containing nucleotides and protein hydrolysis products. Our results showed, however, that these fears were unfounded, and that our mixtures conformed to the addition rule. As is evident from Figs. 4 and 5, the absorption curves obtained for solutions in N $HClO_4$ of mixtures of RNA or DNA with protein hydrolyzate coincide exactly with those given by summation of the separate absorptions of the mixtures.

Our results show that the method of extinction differences may be applied to the determination of nucleic acids, using the specified wavelengths. It then follows from equation (IV), with $E_{260}(P) = 10{,}130$ and the ratio $E_{260}/E_{286} = 2.2$, that the RNA content, expressed as mg-% P, will be given by the formula

$$\text{RNA} = \frac{561\,(E_{260} - E_{286}) \cdot V}{w \cdot l}, \text{ mg \% P},$$

where V is the volume of extract, l is the depth of the layer, and w is the weight of the sample.

For DNA, with $E_{268}(P) = 8782$, and the ratio $E_{268}/E_{284} = 1.79$, the corresponding formula is:

$$\text{DNA} = \frac{800\,(E_{268} - E_{284}) \cdot V}{w \cdot l}, \text{mg-\% P}.$$

It is evident from our results (see table) that the RNA content given by our procedure is considerably lower than when it is derived from measurements of absorption at 260 mμ or from pentose determinations. The results given by the two latter methods for three kinds of tissue exceeded those derived from ΔE measurements by 60-100%. Direct P determinations also gave higher results than did the ΔE method; 31% higher for liver, 22% higher for skin, and 18% higher for Guérin's carcinoma. Our results are in agreement with those reported by Davidson and Smellie [2], that about 25% of the nucleotide P of liver was present in the RNA fraction prepared by the Schmidt-Thannhauser method.

The results obtained by our method for the DNA content are close to those deirved from the diphenylamine reaction for deoxyribose. Taking into account the low sensitivity of the diphenylamine reaction, it may be supposed that the differences between the results of the two procedures are ascribable to the analytical error of the deoxyribose estimation. Moreover, recent reports have shown [5] that the diphenylamine reaction gives lower results when the extraction of DNA is conducted at higher temperatures, and it should also be borne in mind that the deoxyribose content of extracts falls during storage at room temperature.

In all cases, direct determinations of DNA-P gives lower values than those derived from ΔE measurements or from the deoxyribose content. The difference is particularly great for liver, where direct determination of P gives DNA contents lower from 32 and 36% than those derived from ΔE and deoxyribose measurements, respectively. It has been reported [5, 28] that the Ogur-Rosen method for the determination of nucleic acids, which does not involve alkaline hydrolysis, gives higher results for the DNA content than does that of Schmidt and Thannhauser. Some

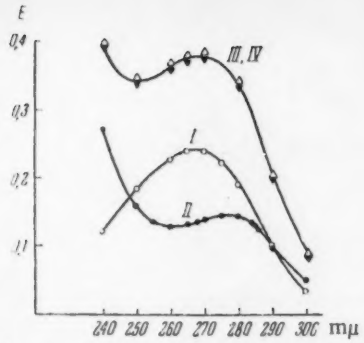

Fig. 5. Additivity of absorption in mixtures of DNA with protein hydrolyzate. I) Absorption curve of DNA after treatment with hot N $HClO_4$; II) absorption curve of alkaline liver protein hydrolyzate; III) absorption curve of mixtures of I and II; IV) curve given by summation of the values of I and II.

authors have hence raised doubts as to the completeness of precipitation of DNA by the Schmidt-Thannhauser method [5]. Our results suggest that possibility that alkaline hydrolysis according to Schmidt and Thannhauser leads to partial dephosphorylation of DNA, part of the phosphate liberated appearing in the RNA fraction. In every case, the excess of P in the RNA fraction was greater than, or equal to, its deficit in the DNA fraction (compare the differences between columns 3 and 6 with those between columns 7 and 10 of the table).

We examined, in a special series of experiments, the effect of varying alkali concentration (from 0.5 to 2 N KOH) on the NA content, as derived from analysis for the three components (P, pentose, and ΔE of bases). We found [10] that variations in the KOH concentration, within the range indicated, did not affect the results obtained.

It is of interest to compare our findings for the RNA and DNA contents of liver with the numerous published values. Most authors using the Schmidt-Thannhauser procedure found that liver contained from 77 to 100 mg-% of RNA-P, and 16 to 26 mg-% of DNA-P, calculated on fresh weight [24, 25]. It now appears that the true RNA contents are considerably lower, and the DNA contents somewhat higher. Moreover, the usually accepted RNA/DNA ratio of 4-5 should be considered as being too high. The values found by the ΔE method are in full accordance with those published by other authors, who used methods involving the preparation of purer NA fractions. Thus the RNA-P values found after mild alkaline hydrolysis of liver tissue (one hour at room temperature) were 50-60 mg-% [11], Manoilov and Orlov [12], who first isolated the RNA in a purified form, found a RNA-P content of about 58 mg-%, and the Belgian workers [5], who separated the impurities from the RNA fraction by adsorption on a Dowex column, reported a RNA content of 2.63 mg per 100 mg of dry, fat-free tissue, which corresponds to about 57 mg-% RNA-P in the fresh tissue. Our mean value of about 53 mg-% of RNA-P (wet weight) (mean of the results for 10 animals, ranging from 49 to 57 mg-%), based on measurements of the ΔE values, as is good agreement with the few published results of analyses in which precautions had been taken to exclude the effects of contaminants. Determinations of RNA based on direct determination of P are also in conformity with our results, if the nonnucleotide P, amounting to about 25%, is taken into account [2]. Applying this correction, the mean value of 77 mg-% RNA-P, derived by us from direct P determinations, is reduced to about 57 mg-%, which is almost precisely the value given by the ΔE method.

The mean DNA-P content given by the ΔE method (25.7 mg-% P, ranging from 19 through 34 mg-%) coincides with the upper limit of the published values based on the Schmidt-Thannhauser method, while the value based on direct P determination corresponds with the lower published values. These differences may be due to partial dephosphorylation of DNA taking place during alkali treatment by the Schmidt-Thannhauser procedure. Further work would, however, be required to elucidate this question.

The tissue components interfering with the UV spectrophotometric determination of nucleic acids are fairly regular contaminants of nucleic acids. In the Schmidt-Thannhauser RNA fractions these contaminants raise the absorption at 260 mμ by 80% for liver, by 103% for skin, by 59% in Guerin's carcinoma, and by 429% in muscle. The chief component of these contaminants, responsible for absorption in the 270-290 mμ band, is the group of aromatic amino acid-tyrosine, tryptophan, and phenylalanine. Of these, the most important are tyrosine and tyrptophan (having the highest extinction values), the absorption maxima of which [16, 29] coincide with that of our alkaline protein hydrolyzates, at 275-280 mμ (Fig. 3). To the presence of these amino acids in all tissue proteins is due to the uniform nature of the absorption curves of the protein hydrolyzates of the different tissues examined by us. It is very probable that the same wavelengths will be suitable for the analysis of the tissues of other organs. It will, however, be necessary, when analyzing any new types of tissue, to select the second wavelength in accordance with the absorption curves of the protein hydrolyzates, as described above.

The constancy of the relation found (judging from the absorption data) between the RNA and the aromatic amino acid contents of a given tissue supports the view expressed by certain authors [12, 30] that some of the linkages between nucleic acids and proteins are covalent bonds involving aromatic amino acids.

We conclude from our results that the published values for the RNA content of various tissues are considerably higher than the true values; this conclusion does not apply to the very small number of quite recent publications [5, 11, 12], in which special precautions had been taken to exclude the effects of contaminants. The effects of contaminants should be excluded from all analyses involving determination of

the nucleic acid contents of tissues. The method described in this paper is a very convenient one, possessing all the advantages of spectrophotometric methods, but not requiring any laborious and time-consuming purification procedures. The method is applicable to the analysis of small amounts of tissue, in which case it is more convient to slice the tissue on a freezing microtome than to homogenize it. The slices are then treated with cold alcohol and ether, acid-soluble substances are extracted with cold 0.2 N $HClO_4$, and the residue is hydrolyzed, after which the further operations on the hydrolyzate are as described above.

SUMMARY

Determination of nucleic acids based on measurements of the phosphorus or pentose contents of tissues, or on their UV absorption, give results higher than the true values,due to the presence of interfering contaminants. A spectrophotometric method, based on measurement of the optical density of RNA and DNA solutions in the presence of these contaminants, at two selected wavelengths. From examination of the absorption curves of hydrolyzates of the nucleic acids and nucleic acid-free proteins of six different tissues, it was found that the optical density of the impure RNA fraction should be measured at 260 and 286 mμ, and of the DNA fraction at 268 and 284 mμ. The RNA content [ϵ (P) = 10,130 and $E_{260}/E_{286} = 2.2$] is then derived from the formula:

$$RNA = \frac{561\,(E_{260} - E_{286}) \cdot V}{w \cdot l}\ \text{mg\%}\ \text{P},$$

and the DNA content [ϵ(P) = 8782 and $E_{268}/E_{284} = 1.79$] from the formula:

$$\text{DNA} = \frac{800(E_{268} - E_{284}).V}{wl}\ \text{mg-\%P},$$

where E is the optical density of the extract at the indicated wavelengths, V is the volume of extract, w is the weight of sample, and l is the length of the light path.

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NUCLEOTIDE COMPOSITION OF RIBONUCLEIC ACIDS OF INTRACELLULAR STRUCTURES OF PLANTS

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Translated from Biokhimiya Vol. 25, No. 1, pp. 160-163, January-February, 1960

Original article submitted August 6, 1959

The study of the nucleotide composition of nucleic acids is of importance in relation to the problems of their specificity, and also for achieving an understanding of their role in cell metabolism. It is known that protein synthesis in cells requires the participation of ribonucleic acids, which in many cases have a definite, species-specific structure. Hence the problem of the specificity of nucleic acids, which is directly implicated with the problem of protein biosynthesis, occupies a prominent place in present-day biochemistry.

The specificity of such complex compounds as are the nucleic acids may present several aspects. Specificity based on the quantitative relationships of the purine and pyrimidine bases (the so-called nucleotide composition) of nucleic acids can be related only to their chemical structure, and the same applies to the sequence of nucleotides in the chain, to the molecular weight of the nucleic acids, to the identity of the nitrogenous bases, and to other such features.

The experimental methods presently available for the study of these problems have been furthest advanced for the determination of the nucleotide composition of nucleic acids. As for the sequence of nucleotides along the chain of the nucleic acid molecule, there are as yet no reliable methods suitable for attacking the problem, in spite of its exceptional importance.

Very little work has been done on the nucleotide composition of the ribonucleic acids of the higher plants [1-11]. Even less is known about the nucleotide composition of the RNA of the intracellular structures of plants [1, 4, 6, 8, 11]. Yet this subject is of considerable interest, inasmuch as it is evident that different intracellular structures participate unequally in the biosynthesis of proteins.

Most of the above cited papers dealt with RNA preparations isolated from plants. It has, however, been shown [12, 13] that this approach to the problem is methodologically unsound, owing to the possibility of incompleteness of extraction of the nucleic acids, as well as of their degradation during the processes of their preparation, and of losses incurred incidentally. The recently published papers of Belozerskii et al. [9, 10] dealt with the over-all nucleotide composition of the RNA of plants of various systematic groups. The experimental material for these studies included seeds, pollen, and thalloid tissue.

The present research was devoted to a study of the overall nucleotide composition of the RNA of intracellular structures of plants.

METHODS

We examined cytoplasmic fractions from 7-10 day pea seedlings, chloroplasts from sunflower, clover, spinach, tobacco, and sugar beet leaves, and leukoplasts from sugar beet roots. The cell particulate fractions were isolated by means of differential centrifugation, except for the case of leukoplasts, which were isolated by Vecher's method [14]. All the operations involved in the fractionation procedures were conducted at 4°. The pea seedlings and the leaves were homogenized in 0.5 M sucrose solution in plastic mortars, so as to minimize mechanical damage to cell structures. The chloroplast fraction was sedimented to 3000 g for 5 min, the cell particularate fraction of the type of mitochondria at 8000 g for 5 min, and the particulate fraction of the microsome type at 45,000 g for 30 min. In order to obtain more homogeneous fractions we also centrifuged the homogenates at 500 g for 5 min and at 17,000 g for 15 min, and discarded the pellets of mixed particles so deposited. Each particulate fraction was washed with 0.5 M sucrose, in order to free it of contaminants. The fractions were fixed in 3-5 volumes of acetone, defatted, and dried in a vacuum desiccator. All fractions were examined under the electron microscope. The dry, fat-free, comminuted products served as the material for the study of the nucleotide composition of plant RNA.

RESULTS

All the cytoplasmic particulates isolated from pea seedlings were found to contain RNA (Table 1).

The data of Table 1 show that the highest RNA contents were in the fraction sedimenting at 45,000 g, containing about 9% of RNA (dry weight), while the lowest values were found in the mitochondria. Thus plant mitochondria resemble those of animal cells in having a low RNA content, amounting to about 1%. The supernatant fluid was found to contain 0.5-0.8% of RNA. This value is, of course, somewhat higher than the real one, because of the presence of microsomes, which are known to be rich in RNA, and which cannot be fully sedimented out after 30 min of centrifugation at 45,000 g. On the other hand,

TABLE 1. RNA Contents of Cell Particulate Fractions from Pea Seedlings*

Fraction	RNA content as % of absolute dry weight
3000 × g 5 min	2.0-4.0
8000 × g 5 min	1.0-1.5
45,000 × g 30 min	8.0-10.0
Supernate	0.5-0.8

* The RNA content of the fractions was derived from their nucleic acid phosphorus contents.

our fraction of microsomal type particulates had a much lower RNA content than has been reported in the literature for plant and animal cells [18, 19]. It is evident that under our experimental conditions we were able to collect chiefly the larger microsomal particles, which have a lower RNA content than do the smaller microsomes which remained in suspension.

We determined the nucleotide composition of the RNA by means of quantitative paper chromatography, followed by spectrophotometric measurements [10, 15, 16]. In some cases we also applied the method of paper electrophoresis [10] to the determination of nucleotide composition. This method did not always give good separation of the mononucleotides of RNA, and, in addition, it often gave very high values for cytidylic acid, evidently as a result of the presence of pigmented substances in the hydrolyzates. The separation of the monoribonucleotides was effected on Leningrad chromatography paper, previously washed in 2 N acetic acid and in water.

We were able, using the above methods, to identify and determine four nucleotides in plant cell particulates, viz., guanylic, uridylic, cytidylic, and adenylic acids.

The results of our studies of the nucleotide composition of the RNA of intracellular structures of plant cells are presented in Tables 2 and 3.

The RNA of the different particulate fractions from pea seedlings conforms to Chargaff's rule, the number of 6-amino groups being equal to that of 6-keto groups, i.e., the sum of the guanylic and uridylic acid contents is equal to that of the adenylic and cytidylic acid contents (Table 2). In all the fractions the sum of the guanylic and cytidylic acid contents exceeds that of the adenylic and uridylic acid contents, showing that the RNA belongs to the GC type, and the purine nucleotides content exceeds that of the pyrimidine nucleotides.

The identical nucleotide composition of all the particulate fractions suggests the absence of organelle specificity of their RNA, at least as regards their nucleotide composition. The results found for the plastids of different plants are given in Table 3.

Like the RNA of the cell particulate fractions of pea seedlings, the RNA of plastids conforms to Chargaff's rule, and belongs to the GC type. The proportion of purine nucleotides is similarly somewhat greater than that of the

TABLE 2. Nucleotide Composition of the RNA of Intracellular Structures Isolated from 7- to 10-day Pea Seedlings

Fraction	Nucleotide content, moles-%				Pur/Pyr	G+C / A+U	G+U / A+C
	G	U	C	A			
3000 × g 5 min.	29,9	20,6	24,2	25,1	1,23	1,18	1,02
8000 × g 5 »	29,8	19,5	24,9	25,7	1,25	1,20	0,98
45000 × g 30 »	29,8	19,9	24,9	25,4	1,23	1,21	0,99
Supernate	29,5	19,5	25,0	25,8	1,24	1,20	0,97

Note: Pur) Purine bases; Pyr) pyrimidine bases; A) adenylic acid; G) guanylic acid; C) cytidylic acid; U) uridylic acid. The figures given are the means of 8-10 separate determinations of each of 2-3 parallel hydrolyzates of the given cell particulate fraction. We took 0.5-3.0 g of material for each hydrolysis, depending on the RNA content of the given fraction.

TABLE 3. Nucleotide Composition of the RNA of Plastids of Different Plants

Material	Nucleotide content, moles-%				Pur/Pyr	G+C / A+U	G+U / A+C
	G	U	C	A			
Chloroplasts:							
Pea	29,9	20,6	24,2	25,1	1,23	1,18	1,02
Sunflower	30,3	20,0	24,9	24,7	1,22	1,23	1,00
Clover	29,4	19,9	25,0	25,6	1,23	1,20	0,97
Spinach	30,7	19,2	24,3	25,6	1,29	1,22	0,99
Tobacco	29,7	20,2	24,7	25,1	1,22	1,19	1,00
Sugar beet	29,4	20,2	25,3	24,8	1,20	1,23	0,98
Leukoplasts from sugar beet root	30,1	19,5	25,0	25,3	1,24	1,23	0,98

Note: The abbreviations are as in Table 1.

pyrimidine nucleotides. The nucleotide compositions of the RNA of the plastids of different plant species resemble each other closely. In all cases, the guanylic acid content exceeded the adenylic acid content, amounting to about 30%; the uridylic acid content was lower than that of cytidylic acid, being about 20%. Cytidylic and adenylic acid were present in about equal amounts. The nucleotide compositions of the RNA of different classes of plastids (chloroplasts and leukoplasts) closely resembled each other. A comparison of the nucleotide composition of the RNA of different cell particulate fractions, of the plastids of different plants, and of higher plants, as published in the literature [4, 9, 10], shows clearly that there is little variation in the nucleotide composition of the RNA of higher plants, and that it reflects the conservative nature of this feature of plant metabolism.

We have been able to find a single paper [11] reporting that formation of the photosynthetic apparatus in etiolated Euglena cells is associated with modification of the nucleotide composition of the RNA of the cells, involving a slight rise in the adenylic acid content, with a corresponding slight fall in the cytidylic acid content. This finding still awaits confirmation, however.

It may be concluded from our results and from published data that the nucleotide content of RNA does not reveal the nature of the specificity of these substances, and that other methods will be necessary to achieve a solution of this problem, such as, for example, the determination of the sequence of nucleotides in the RNA chain.

SUMMARY

A study has been made of the content and nucleotide composition of the RNA of intracellular structures of plants. These included cell particulates of pea seedlings, chloroplasts from sunflower, clover, spinach, tobacco, and sugar beet leaves, and leukoplasts from sugar beet roots. The particulate fractions were isolated by means of differential centrifugation of homogenates in 0.5 M sucrose solution, at 3000 g for 5 min, at 8000 g for 5 min, and at 45,000 g for 30 min. The nucleotide composition of the RNA was determined by methods involving paper chromatography, electrophoresis, and spectrophotometry.

The data presented in the tables represent the means of 8-10 determinations of nucleotide composition of 2-3 parallel hydrolyzates of each given particulate preparation. The RNA of all the cell particulates was found to conform to Chargaff's rule, and to belong to his GC type.

Practically identical results were obtained for the nucleotide composition of the cytoplasmic particulates of pea seedlings, which also closely resembled that found for the plastids of a number of plants.

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* Original Russian Pagination. See C.B. translation.

TRANSAMINATION OF AMINO ACIDS OF THE ORNITHINE CYCLE IN PEA AND WHEAT SEEDLINGS

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Translated from Biokhimiya Vol. 25, No. 1, pp. 164-167, January-February, 1960

Original article submitted August 24, 1959

Researches on the ornithine cycle in leguminous plants have shown [1-3] that incubation of plant homogenates with ornithine and citrulline, or introduction of these substances into living plants, leads to production of glutamic acid, glutamine, and asparagine, in addition to metabolites of the ornithine cycle.

The present research is devoted to an investigation of the possibility of transamination of ornithine, citrulline, and arginine with α-ketoglutaric acid (α-KG) and oxalacetic acid (OAA). We would find very few references in the literature to this type of metabolic pathway in plants. This may be due to the circumstance that the presence of ornithine and citrulline in many plants was only recently established.

Miettinen and Virtanen [4] have shown that alder leaves contain an enzyme catalyzing transamination between citrulline and α-KG. Transamination was, however, more active with other amino acids, such as alanine and γ-aminobutyric acid, than with citrulline. This enzyme was not found in alder tubers. These authors suggested that citrulline synthesized in alder tubers is transported to the leaves, where it undergoes transamination with α-KG.

Wilson, King, and Burris [5], who treated etiolated white lupin seedlings with C^{14}-α-KG and ornithine or arginine, found that C^{14} was present in glutamic acid.

MATERIALS AND METHODS

We used 3-20-day pea and wheat seedlings, grown in water.

Extraction of the material, and dialyses, were conducted in the cold room, at 4-5°. The extracts were prepared from 10-30 g portions of the green parts of the plants. In addition, we examined dialyzates of pea cotyledons in two of the experiments. The material was first frozen in Dewar vessels with dry ice, for 1 hour, and was then ground up in a porcelain mortar with 2-3 volumes of Tris buffer (tris-hydroxymethylaminomethane) of pH 8.0. The amount of buffer solution taken was such as to give a tris concentration in the extracts of 0.10-0.15 M. The suspensions were centrifuged at 4000-5000 rpm for 10 min, and the sediments were discarded. The extracts, volume about 20-30 ml, were dialyzed against 1 liter of 0.005 M tris of pH 8.0, using a mechanical stirrer. The dialyzing solution was changed 4-6 times. The duration of dialysis was 20-24 hr. After completion of dialysis, the contents of the sac were transferred to a small conical flask, 1 ml was taken for total nitrogen determination, and the remainder was stored in the refrigerator.

Substrates used. The L- and D, L-amino acids were supplied by Hoffman-La Roche, Nutritional Biochemicals Corporation, and British Drug Houses (B.D.H), and the keto-acids by Hoffman-La Roche, B.D.H., and the Nitrogenous Metabolism Laboratory, A. N. Bakh Institute of Biochemistry, Academy of Sciences USSR; pyridoxal phosphate was supplied by B.D.H.

The substrates were dissolved in freshly redistilled water, and the pH of the solutions was adjusted to 7.5-8.0 by addition of 7% NaOH.

The experimental systems contained the following amounts of substrates: L-amino acids 20 μM, DL-amino-acids 40 μM (0.1 ml), keto acids 30 μM (0.1 ml) pyridoxal phosphate about 40 γ (1 drop). The systems also contained 1 ml of dialyzed extract and 0.2 ml of 0.5 M tris or phosphate buffer of pH 8.0. The systems were contained in conical calibrated 10 ml centrifuge tubes. The systems containing pyridoxal phosphate were incubated at 37° for 30 min before adding the remaining substrates. The volume of all the systems was made up to 1.5 ml with redistilled water, and the systems were incubated at 37° for 3 hours from the time of mixing the components.

The control systems consisted of dialyzed extract, boiled dialyzed extract + the full set of substrates, dialyzed extract + keotacid, and dialyzed extract + keto acid + + pyridoxal phosphate.

After incubation, the reaction was arrested by adding ethanol to a final concentration of 70-75% v/v, the solution was placed in a refrigerator for precipitation of proteins, and was then centrifuged at 6000 rpm for 15 min. The transparent supernate was transferred to a calibrated conical 10 ml centrifuge tube, and evaporated to dryness in a vacuum desiccator over NaOH. The dry residue was dissolved in 1 ml of 48% ethanol and 0.1-0.2 ml portions were taken for paper chromatography.

For chromatography we used slow Leningrad paper or Whatman No. 1 paper, previously treated with phosphate buffer of pH 12, according to MacFarren [6], and the

amounts of glutamic and aspartic acids present in the spots were determined by the method of Kretovich and Uspenskaya [7]. The results are presented as γ of amino acid per mg of N in the dialyzed extract.

RESULTS

The results are presented in Table 1.

The data of Table 1 show that pea and wheat seedlings possess enzyme systems capable of transferring amino groups from ornithine, citrulline, and arginine to α-KG. The transamination system is most active with ornithine, somewhat less so with citrulline, and least active with arginine.

The transamination reactions effected by pea cotyledons were the same as by the seedlings, but they proceeded at slower rate. Pea seedlings older than 16 days showed only trace activity for transamination of arginine with α-KG. The transamination activity of wheat seedlings was less than that of peas.

Since the tris buffer used in these experiments contains an amino group, we repeated the experiments, using two extracts prepared in parallel, taking tris as the buffer for one, and phosphate for the other. The dialyzed extracts were incubated in parallel, in systems such as described above. Two such experiments were performed on pea seedlings, and they gave concordant results. The results of one of these experiments are presented in Table 2. They show that there was no significant difference between the activities of the enzyme systems with tris or phosphate buffer.

An experiment was performed in which we incubated dialyzed extract with alanine, α-KG, and pyridoxal phosphate, in order to be able to compare the reactivity of this amino acid with that of those of the ornithine cycle (Table 2). As is evident from this table, the rate of transamination of alanine with α-KG is overwhelmingly greater than of the other amino acids examined.

In addition to the experiments with α-KG, we performed a few experiments on transamination of amino acids of the ornithine cycle and oxalacetic acid. These experiments gave no evidence of any transamination activity between amino acids of the ornithine cycle

TABLE 1. Transamination of Amino Acids of the Ornithine Cycle with α-Ketoglutaric Acid (glutamic acid synthesized, as γ per mg N of the dialyzed extract)

Plant	Part of plant	Age of plant, days	Substrate		
			ornithine	citrulline	arginine
Peas, Rannyi variety	Seedlings	3	9,0	7,3	5,8
» » »	Cotyledons	3	5,1	2,6	1,7
» » »	Seedlings	8	11,2	5,9	2,8
» » »	»	11	13,0	13,0	7,0
» » »	Cotyledons	11	8,0	6,4	3,4
Peas, Chaika variety	Seedlings	13	13,0	9,0	4,0
» » »	»	20	11,0	8,0	Traces
Wheat, Moscow winter	»	4	8,0	4,2	Traces
>> >> >>	»	9	3,0	0,6	Traces

Note: The figures represent the differences between the glutamic acid contents of the systems: experimental (dialyzed extract + α-KG + appropriate amino acid) and control (dialyzed extract + α-KG). The glutamic acid content of the controls did not exceed 10-50% of that of the experimental systems.

TABLE 2. Effects of Different Buffers and of Pyridoxal Phosphate on Transamination Between Amino Acids of the Ornithine Cycle and Alanine and α-Ketoglutaric Acid (16-day pea seedlings (Maiskii variety) were taken for the experiments. Glutamic acid is expressed as γ per mg N of the extracts)

Composition of the systems	Phosphate buffer, pH 8.0		Tris buffer, pH 8.0	
	I	II	I	II
Dialyzed extract	15,0 —	— —	14,5 —	— —
Dialyzed extract + α-KG	27,5(12,5)	32,0 —	26,8(12,3)	35,0 —
Dialyzed extract + α-KG + + ornithine	60,0(32,5)	70,0(38,0)	60,3(33,5)	70,0(35,0)
Dialyzed extract + α-KG + + citrulline	42,3(14,8)	48,0(16,0)	46,0(19,2)	52,0(17,0)
Dialyzed extract + α-KG + + arginine	29,0(1,5)	33,3(1,3)	28,0(1,2)	37,0(2,0)
Dialyzed extract + α-KG + + alanine	536,7(509,2)	711,6(679,6)	605,0(578,2)	794,0(759,0)

Note: The figures in parentheses express the rise in glutamic acid content over that of the controls. I) Without pyridoxal phosphate; II) with pyridoxal phosphate.

and oxalacetic acid. We found, however, that in all the experiments oxalacetic acid reacted nonenzymatically with ornithine, as a result of which two spots appeared on the chromatograms, both having low R_f values. The R_f of the slower-running spots is close to that of aspartic acid. No such effect was found when oxalacetic acid was incubated with systems containing the other amino acids of the ornithine cycle.

DISCUSSION OF RESULTS

The results of our experiments show that the plants investigated are capable of synthesizing glutamic acid by transamination of ornithine, to a lesser extent, of citrulline, and least of all, of arginine, with α-KG. This process proceeds far less actively than with alanine.

Wilson, King, and Burris [5] found that transamination between arginine and α-KG proceeded more actively in etiolated white lupin shoots than did the corresponding reaction with ornithine. In our experiments the most active exchange was found with ornithine, and the least active with arginine.

Fincham et al [8, 9], examining transamination between ornithine and α-KG in Neurospora, came to the conclusion that a specific enzyme was responsible for the reaction, since it was the δ-amino group of ornithine which was transferred, and not the α-amino group, as is the case in most transamination reactions.

The products of such an exchange reaction with α-KG are glutamic acid and glutamic γ-semialdehyde, which is, according to Vogel and Davis [10] (Escherichia coli), in equilibrium with its cyclic form (Δ'-pyrroline-5-carboxylic acid), which is, in turn, readily reduced enzymatically to proline.

The rate of the transamination reaction with amino acids of the ornithine cycle is much lower than that found in our earlier experiments [1-3], in which the glutamic acid and glutamine spots of the chromatograms were much more intense. It may hence be concluded that the exchange reaction examined in the present paper represents only one of the possible pathways of synthesis of glutamic acid from ornithine and other amino acids of the ornithine cycle.

It is possible that the reversible synthesis of ornithine from glutamic acid, with intermediary formation of N-acetylglutamic acid and, and N-acetylornithine, reported by Vogel and Bonner [11] to take place in Escherichia coli, also proceeds in higher plants. This possibility is supported by the work of Kasting and Delwiche [12] and of Coleman and Hegarty [13] on the metabolism of C^{14}-labeled glutamic acid and glutamine.

SUMMARY

Pea and wheat seedlings (3-20-day-old) contain enzyme systems capable of catalyzing transamination reactions between ornithine, citrulline, and arginine and ketoglutaric acid. The greatest activity was shown by ornithine, and the least by arginine. The rate of these transamination reactions was very low, as compared with that found using alanine. The reactions proceeded at the same rate with tris buffer or with phosphate buffer.

The transamination activity of the green parts of pea seedlings was higher than that of the cotyledons or of wheat sprouts.

Enzymatic transamination between ornithine and oxalacetic acid was not observed. A nonenzymatic reaction between the two substrates takes place, however, yielding a product had a R_f value close to that of aspartic acid.

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STUDY OF THE FORMATION OF CHLOROPHYLL IN HOMOGENATES OF ETIOLATED LEAVES USING A FLUORESCENCE SPECTROPHOTOMETRIC METHOD

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Translated from Biokhimiya Vol. 25, No. 1, pp. 168-179, January-February, 1960

Original article submitted August 24, 1959

There is extensive experimental support for the view that protochlorophyll, formed in etiolated plants, is a direct precursor of chlorophyll. Detailed investigations of the formation of chlorophyll in living, uninjured leaves, which have recently been carried out with the aid of absorption spectroscopy [1] and of fluorescence spectrophotometric methods involving deep cooling of living leaves [2], have, however, shown that a series of intermediate stages are involved in the process of conversion of protochlorophyll into chlorophyll in living leaves.

It was shown, that the same time, that protochlorophyll and freshly formed chlorophyll exist in two forms, differing in the presence or absence of the phytyl side-chain [3-6]. Esterification with phytol may be achieved after the stage of reduction of the precursor pigment, But, as will be shown in the following survey, no correspondence has yet been established between the various forms of pigment detected by spectroscopic methods and the various chemically different intermediate forms.

An extremely labile and photochemically active form of protochlorophyll, with an absorption maximum of 650 mμ, when in the native state [1], and with a fluorescence maximum at 655 mμ [2], has been found to participate in the first photochemical stage of formation of chlorophyll. The discovery of this form of protochlorophyll in living etiolated leaves, with an intact native structure, explains the formerly uninterpreted shift in the maximum of the "action" spectrum of formation of chlorophyll, toward the longer wave region (650 mμ) [7, 8], as compared with the position of the absorption maximum of protochlorophyll in solutions (625-630 mμ). This active form of protochlorophyll reminds one of Lyubimenko's "chlorophyllogen" [9].

The active form of protochlorophyll disappears within the first few minutes of the action of light on etiolated leaves, giving rise to a new intermediate form of chlorophyll with an absorption maximum at 684 mμ [1] and a fluorescence maximum at 690 mμ [2]. This form undergoes transformation during the dark period, giving rise to other intermediate forms, and finally to chlorophyll, with an absorption maximum at 676 mμ [1] and a fluorescence maximum at 680 mμ [2]. The entire process of consecutive transformations of pigments proceeding in intact living leaves may be represented by the following scheme [1, 2]: $P_{655(650)} \rightarrow C_{690(684)} \rightarrow C_{680(670-672)} \rightarrow C_{684-686\,(676-678)}$ where P stands for protochlorophyll, and C for chlorophyll; the subscripts refer to the fluorescence maxima and to the corresponding absorption maxima (in parentheses).

Krasnovskii and Kosobutskaya [10, 11] have shown that homogenates – colloidal solutions of etiolated leaf material – retain the capability of forming chlorophyll, and that transformation of protochlorophyll into chlorophyll takes place when such suspensions are exposed to light. In their first paper, these authors used colloidal solutions of etiolated bean leaf material in phosphate buffer, illumination of which gave rise to primary chlorophyll, with an absorption maximum at 670 mμ. This form of chlorophyll is rapidly decolorized with continuing illumination. Smith et al. [12], who studied this reaction in glycerol extracts of etiolated leaves, found a different form of chlorophyll, with an absorption maximum at 680 mμ, as the primary product of the pigment precursor. On standing, the newly formed chlorophyll underwent transformation to a form with maximum absorption at a shorter wavelength of 672 mμ.

Smith found that a pigment-protein complex, termed 'holochrome', retained its activity when purified by fractional precipitation with ammonium sulfate [13].

The object of the present research was to study the mechanism of formation of chlorophyll in homogenates of etiolated leaves, and to ascertain whether, and in what respects, this process differs from that taking place in intact living leaves. We examined the processes taking place during illumination of the homogenates, without attempting to make any sharp distinction between the light and the dark processes. We applied a fluorescence spectrophotometric method, at very low temperatures. The sensitivity of the fluorescence spectrophotometry method exceeds that of absorption spectroscopy. Photobiochemical processes are retarded at the low temperatures applied by us, thus enabling us to study stages of the process which proceed very rapidly at ordinary temperatures.

We examined the fluoresence spectra of homogenates not exposed to light, the sequential spectral changes taking place on illumination, and the effects of various factors on the fluorescence of homogenates before and after

exposure to light, and on the course of the fluorescence changes following illumination.

METHODS

Etiolated leaves from 12- to 15-day bean plants, var. Severnaya Svezda, grown at 20-23°, were ground up in the dark with 20 ml of phosphate buffer of the appropriate pH, at 0°. The suspension was strained through muslin, to remove the coaser fragments of cell debris.*

A number of portions of homogenate were measured out, by filling to the brim a glass 3 ml beaker. All these operations were carried out in absolute darkness, by touch only, which required a fair amount of practice on the part of the experimenter.

The samples were kept in the dark at 0°, and each series of experiments were performed on the same batch of homogenate.

Each 3 ml portion was subjected to the appropriate treatment (illumination, heating, addition of solvents), after which we added 5 ml of glycerol, for clarification and stabilization of the suspensions. The suspensions in buffered glycerol were stirred thoroughly, and were then immersed in liquid nitrogen in a Dewar flask, for a few minutes. This yielded a transparent frozen mass.

The tubes containing frozen homogenate were transferred to a holder (a "cold conductor"), consisting of a copper tube closed at the lower end by a copper rod pressed in, with a small window cut through the central part of the tube, serving for illumination of the material and for transmission of fluorescent light. The inside diameter of the copper tube corresponded with the outside diameter of the test tube. Cooling of the systems during measurement of fluorescence was effected by immersing the lower end of the inserted rod in liquid nitrogen in a Dewar flask. A thin layer of antifreeze solution (5% alcoholic glycerol) was applied to the outer surface of the tube, by means of a brush, immediately before taking photographs, in order to prevent misting.

Fluorescence was excited by illumination from a SVDSh-250 quartz-mercury vapor lamp, filtered through water and through SZS-9 and SZS-8 light filters.

The equipment described by Litvin and Krasnovskii [2] was used for recording the fluorescence spectra. These were recorded photographically on "Paninfra" film, which has high sensitivity within the spectral region concerned (620-720 mμ).

The absence of reflected exciting light from the fluoresence region was checked by experiments in which a red KS-11 light filter was placed in front of the spectrograph slit.

The usual exposure time was 2.5-3 min with an incident slit width of 0.1 mm. Such short exposure times reduced to a minimum the action of the exciting radiation, and the narrowness of the slit assured good spectral definition. We measured the position of the fluorescence bands, with an accuracy of ± 1 mμ, and the density of the image, for a qualitative assessment of the intensity of the fluorescence maxima, with the aid of a nonrecording MF-2 microphotometer. The fluorescence spectra were recorded on a MF-4 recording microphotometer.

EXPERIMENTAL

Fluorescence Spectra of the Initial (Not Illuminated) Homogenates of Etiolated Bean Leaves

The homogenates were made in M/15 phosphate buffer solutions of pH 4.5, 6.5, and 8.5. The fluorescence spectrum of the homogenate at pH 8.5 (Fig. 1, Curve 1) shows two major maxima, at 635 and 655 mμ, and two minor maxima, at 687-689 and at 705-707 mμ. The most intense maximum is at 655 mμ. At pH 6.5 the intensity of the maximum at 635 mμ is smaller, appearing only as a shoulder of the 655 mμ maximum. At pH 4.5 the short wave maximum consists of a number of bands in the region from about 628-642 mμ.

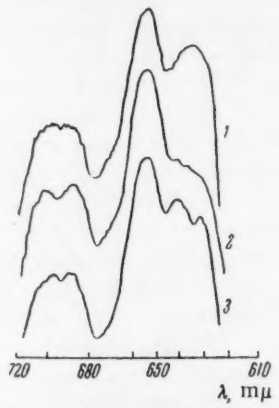

Fig. 1. Fluorescence spectra of unilluminated homogenates of etiolated bean leaves at various pH values. 1) pH 8.5; 2) pH 6.5; 3) pH 4.5.

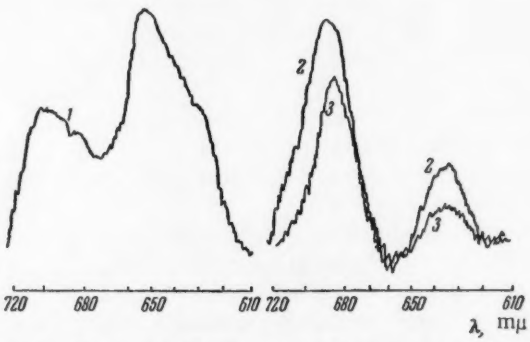

Fig. 2. Changes in fluorescence of homogenates during illumination under aerobic conditions at pH 6.5. 1) Before illumination; 2) after 15 sec of illumination. 3) after 5 min of illumination.

* Centrifugation at 15,000 g did not affect the capability of the supernate to synthesize chlorophyll.

Experiments were performed on homogenates in which glycerol was replaced by an equal volume of buffer solution. We found that the fluorescence spectra were the same in the presence or absence of glycerol, the position of the maxima being the same, as were also their relative intensities. We hence concluded that glycerol did not affect the fluorescence phenomena, and that its use for the purposes of reducing light-scattering and of stabilizing the suspensions is justifiable.

Changes in Fluorescence of Illuminated Homogenates

The homogenates were exposed to light from a daylight lamp (strength of illumination about 2000 lux), at 22-24°. Figure 2 shows the fluorescence spectra of the homogenate at pH 6.5 before exposure (curve 1), after 15 sec of illumination (curve 2), and after 5 min of illumination (curve 3).

Profound changes appear in the spectrum within the first few seconds of exposure to light (under aerobic conditions). The height of the maximum at 655 mμ falls abruptly after 1 sec of illumination, and this maximum disappears completely after exposure for 1 min; only a slight fall was found in the intensity of the maximum at 655 mμ under these conditions. Parallel with the fall in intensity of the maximum at 655 mμ we observed a sharp rise in fluorescence at 687-689 mμ, but after a few minutes this maximum shifted towards the shorter wavelength region, at 680 mμ. The intensity of both maxima fell with further illumination, as a result of bleaching of the pigment in the presence of atmospheric oxygen. The appearance of the maximum at 687-689 mμ, with simultaneous fading of fluorescence of 655 mμ, was found to take place under very feeble, twilight illumination.

The maximum at 687-689 mμ developed slowly in frozen homogenate exposed to prolonged illumination; this finding is in agreement with that of Smith [14], who found that etiolated leaves turned green when illuminated at −70°. Bearing this in mind, we took the first spectrum recorded as the initial one.

Effect of pH on the Fluorescence Changes Occurring during Illumination of Homogenates under Aerobic Conditions

The sequence of spectral changes taking place during illumination of homogenates at pH 4.5, 6.5, and 8.5 is the same in all cases, but the rate of the reactions, and the shift in the position of the main maximum the short wavelength end of the spectrum, are different.

Figure 3 represents the changes in the position of the long wavelength maximum following exposure to light at different pH values of the homogenates. The origin of all the curves corresponds with the position of the long wavelength fluorescence maximum during the first second of exposure to light. The rate of displacement of the maximum, and the extent of this shift, were greatest for suspensions of pH 8.5 than at other pH, which is evidence

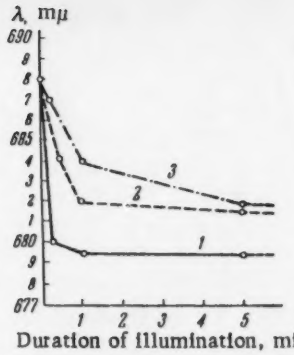

Fig. 3. Displacement of the long wavelength fluorescence maximum during illumination of homogenates at different pH values. 1) pH 8.5; 2) pH 6.5; 3) pH 4.5.

of the higher velocity of the pigment transformation processes at this pH.

Comparison of the Fluorescence Changes Occurring during Illumination of Etiolated Leaves and of Their Homogenates

Etiolated bean leaves were illuminated under the same conditions as for the homogenates. Homogenates of the illuminated leaves were prepared, and their fluorescence spectra were compared with those of illuminated homogenates.

The density of the photographic film (figures in parentheses) at the fluorescence maxima (λ, mμ) indicated are presented in the table, for illuminated homogenates and for intact leaves.

Illumination of homogenates

Before exposure to light	634(56)	656(75)	690(32)	710(16)
5 sec exposure	634(50)	655(28)	687(173)	— —
30 sec exposure	635(49)	655(14)	684(142)	— —
5 min exposure	635(36)	— —	682(77)	— —
30 min exposure	— —	— —	—	— —

Illumination of intact leaves

Before exposure to light	634(56)	656(65)	690(32)	710(16)
5 sec exposure	—	—	690(170)	—
30 sec exposure	—	—	690(250)	—
5 min exposure	—	—	690(130)	—
30 min exposure	—	—	684(68)	—

The long wavelength fluorescence maximum (688-690 mμ) persisted longer in intact leaves than in homogenates. Very short exposures of homogenate caused displacement of this maximum to the left, while longer exposures did not give this effect in intact leaves. A second difference was in the relative intensities of the fluorescence maxima, which were displaced toward the red end of the spectrum (688-690 mμ) in the case of leaves. The maximum at 635 mμ disappeared completely after exposure of whole leaves for only a few seconds, whereas after equal times of exposure of homogenates the fluorescence

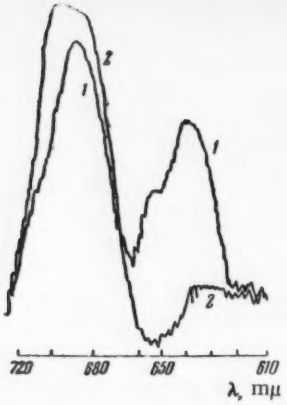

Fig. 4. Fluorescence spectra of homogenate (pH 6.5) illuminated for 5 min (1), and of homogenate prepared from etiolated leaves which had been illuminated for 5 min (2).

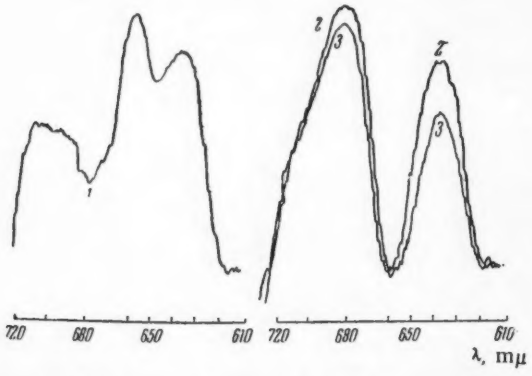

Fig. 5. Changes in fluorescence of homogenate (pH 8.5) during prolonged exposure to light under anaerobic conditions. 1) Initial homogenate, before illumination; 2) after 20 min exposure to light; 3) after 1 hour 20 min exposure to light.

maximum at 635 mμ was still of comparable intensity to that at 688-690 mμ.

Figure 4 presents photomicrograms of fluorescence spectra of homogenate illuminated for 5 min (curve 1), and of homogenate of intact etiolated leaves which had been illuminated under the same conditions for the same length of time (curve 2).

Changes in Fluorescence during Illumination of Homogenates under Anaerobic Conditions

In this series of experiments we placed 3 ml portions of etiolated leaf homogenate at pH 8.5 into Thunberg tubes, from which air was evacuated for 5-7 min by means of an oil vacuum pump, shaking the tube during evacuation.

The changes caused by brief exposures to light (from a few seconds to a few minutes) of homogenates of etiolated leaves under anaerobic conditions resembled those described above for aerobic conditions.

The fluorescence spectra found after prolonged illumination resembled those found after short exposure to light. The position of both the maxima, their relative intensity, and the general contours of the spectra were not affected by prolonged exposure to light, because destructive oxidation of the pigment does not take place (Fig. 5).

The appearance of an intense maximum at 680 mμ, assocated with a reduction in the intensity of the 635 mμ maximum, are thus the final stage of the sequence of fluorescence spectrum changes taking place during illumination of homogenates. Prolongation of illumination beyond this stage leads, in the presence of air, to bleaching of the pigment, with which is associated weakening of its fluorescence.

Effects of Various Factors on the Fluorescence of Unexposed Homogenates of Etiolated Leaves

Effect of heat. Portions (3 ml) of homogenate were heated at 40, 50, 60, 80, and 100° for 2 min, in total darkness. Figure 6 shows microphotograms of unilluminated homogenates heated at 40° (curve 2) and at 50°(curve 3), and of unheated homogenate (curve 1).

Thermal treatment caused profound changes in the fluorescence spectra; even at 36-40° the maximum at 655 mμ was greatly reduced in intensity, and that at 635 mμ heightened. The maxima at 688 and 707 mμ were replaced by a single maximum at 698 mμ.

Heating at 50° caused total disappearance of the maximum at 655 mμ, with further rise in the height of the fluorescence maxima at 635 mμ and at 698 mμ; heating at 60-80° caused only slight further increase in the intensity of fluorescence at 635 mμ, and heating at 100° led to a general fall in the intensity of fluorescence. These effects are shown graphically in Fig. 7, in which are represented the changes in intensity of the fluorescence maxima of unilluminated homogenates following thermal treatment. Similar results were obtained with etiolated intact leaves. The results found for homogenates heated at 100° are in accordance with Virgin's finding [15] that heated etiolated leaves give a fluorescence maximum at 630 mμ.

Effects of different solvents. Equal volumes (3 ml) of homogenate were added, in total darkness, to a series of test tubes containing solvent-buffer solution mixtures in proportions such that the final solvent content of the systems amounted to 1, 2.5, 5, 10, 20, and 30%. After thorough mixing, the systems were kept at 0° in the dark for a few minutes, following which they were frozen, and the fluorescence spectra were recorded.

The solvents caused profound changes in the fluorescence spectra, analogous to those produced by thermal treatment. The maximum at 655 mμ fell, and that at 635 mμ rose, in the presence of 1% of pyridine. With 2.5% of pyridine, the maximum at 655 mμ was absent, while that at 635 mμ rose steeply, and the two peaks at 688 and 705 mμ were replaced by a single peak at 696 mμ. Further increase in the pyridine concentration caused

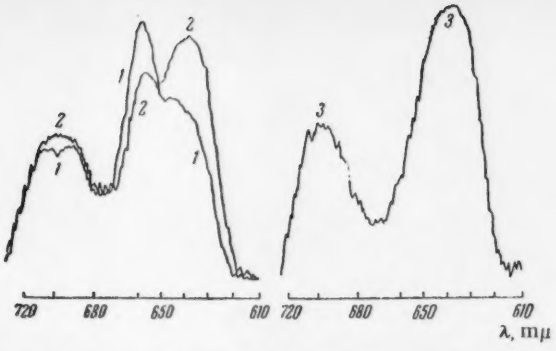

Fig. 6. Effect of heating at various temperatures on the fluorescence of unilluminated homogenate (pH 6.5). 1) Original homogenate, before heating; 2) after heating at 40°; 3) after further heating at 50°.

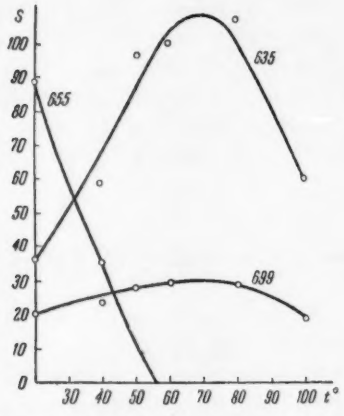

Fig. 7. Effect of thermal pretreatment on the relative intensity of the fluorescence maxima of unilluminated homogenates.

a general increase in the intensity of fluorescence.

Alcohol gave similar changes in the fluorescence spectrum to those observed with pyridine, although to a smaller degree. Significant reduction in the intensity of the maximum at 655 mμ was not achieved below 10-20% of alcohol, and the total abolition of the maximum at 655 mμ, with steep increase in the intensity of fluorescence at 635 mμ, took place when the alcohol concentration reached 30%. We found that the general intensity of the fluorescence was considerably lower in 20% than in 10% alcohol; this effect was evidently due to coagulation of protein.

These solvent effects are in conformity with those reported by Kosobutskaya and Krasnovskii [16] for the effects of solvents on the photochemical activity and spectral properties of green leaf homogenates, and by Litvin and Krasnovskii [17] for the action of solvents on etiolated leaves.

Effects of strongly acid or strongly alkaline media. Etiolated leaf homogenates were prepared in the usual way, in buffer solutions of pH 2.5 and 10; the buffers were prepared by adding 0.1 N HCl to M/15 K_2HPO_4 (pH 2.5), or 0.1 N NaOH to Na_2HPO_4 solution (pH 10).

Under these conditions the maximum at 655 mμ was absent, and there was a considerable fall in the general level of intensity of fluorescence, which showed a weak maximum at 635 mμ, and a barely perceptible maximum in the long wavelength region, at about 702 mμ in strongly acid, and at about 696 mμ in strongly alkaline, homogenates. These effects are probably associated with denaturation of proteins.

Changes in the Fluorescence of Homogenates of Etiolated Leaves Exposed to Light after the Action of Various Factors

Those factors which bring about the abolition of the fluorescence maximum at 655 mμ also abolish the capacity of chlorophyll formation in the homogenates. To such factors belong: heating at over 50°, the presence of more than 1% of pyridine, or of 20% of alcohol, in the medium, and the use of media of very low (2.5) or high (10) pH. When homogenates which had been exposed to the action of such factors were illuminated under arobic conditions, the effect was to give a general lowering of the intensity of fluorescence, due to bleaching of the pigments. Under such conditions the reactions leading to the production of chlorophyll could not be observed.

With less drastic treatment, not leading to the total abolition of the maximum at 655 mμ, the homogentates retain the capacity of forming chlorophyll. The general nature and the sequence of fluorescence changes taking place after illumination resembled those found for untreated homogenates, but the process stopped short of its final stage (formation of chlorophyll giving a fluorescence maximum at 680 mμ), terminating with formation of pigment forms giving maximum fluorescence at 684-682 mμ.

The terminal stage of the sequence of spectral changes observed during aerobic illumination of homogenate preheated at 40° was the appearance of a maximum at about

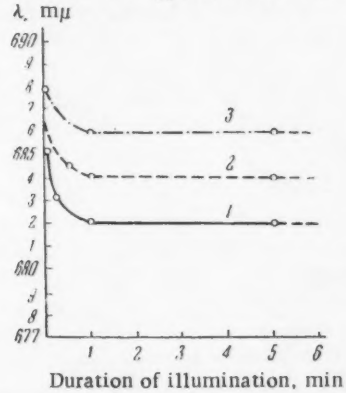

Fig. 8. Shift of the position of the long wavelength fluorescence after illumination of homogenates containing 1 % of pyridine (1), 10% of alcohol (2), or 20% of alcohol (3).

683 mμ, with weakening of the fluorescence maximum at 635 mμ. The general level of intensity of fluorescence fell over the period of illumination. Illumination of homogenates containing 1% of pyridine gave the same final product as in the heated homogenate, with a fluorescence maximum at 682 mμ, and with a fall in fluorescence at 635 mμ. The terminal change seen during illumination of homogenate containing 10-20% of alcohol was the appearance of a maximum at 684-685 mμ, the intensity of which fell after 5 min of illumination (due to bleaching of the pigment). Figure 8 shows the shifts in the position of the long wavelength fluorescence maximum, taking place during illumination of etiolated leaf homogenates containing 10 and 20% of alcohol, and 1% of pyridine.

Fluorescence Changes Taking Place during Illumination of Homogenates Containing 60% Glycerol

In this series of experiments we added 5 ml of glycerol to 3 ml of homogenate, in total darkness, and, after thorough mixing, we exposed the homogenate to light, as described above (in the preceding experiments glycerol was added to the illuminated homogenates).

The maximum at 655 mμ disappeared completely after a few seconds of illumination, and the intensity of fluorescence at 635 mμ diminished. At the same time, an intense maximum appeared at 688 mμ, the position of which was not affected by futher illumination. Further illumination (10 min or more) caused a fall in the general level of intensity of fluorescence, with a considerable fall in the intensity of the peak at 688 mμ, but without any shift in its position. It may hence be concluded that the appearance of the maximum at 688 mμ, with weakening of that at 635 mμ, represents the terminal stage in the sequence of pigment transformations proceeding in buffer-glycerol suspensions during their illumination. In other words, glycerol stabilizes the fluorescence maximum at 688 mμ, thus inhibiting further transformations involving a shift toward the short wavelength end of the spectrum.

Effects of Certain Forms of Treatment of Illuminated Homogenates of Etiolated Bean Leaves on Their Fluorescence

We examined the effects of heat and of pyridine on the initial, intermediate, and final stages of pigment transformation in illuminated homogenates, characterized by consecutive changes in the position of the long wavelength fluorescence maximum from 688 to 684, to 680 mμ.

Pyridine was added to the homogenates, to a final concentration of 2.5 and 5%. Heated systems were prepared by immersing the homogenates for 2 min in boiling water. The systems were prepared in accordance with the conditions found to be necessary for the formation of the various forms of chlorophyll; the form giving maximum fluorescence at 688 mμ by illumination for 1 min in a medium containing 60% of glycerol (pH 6.5 and 8.5); at

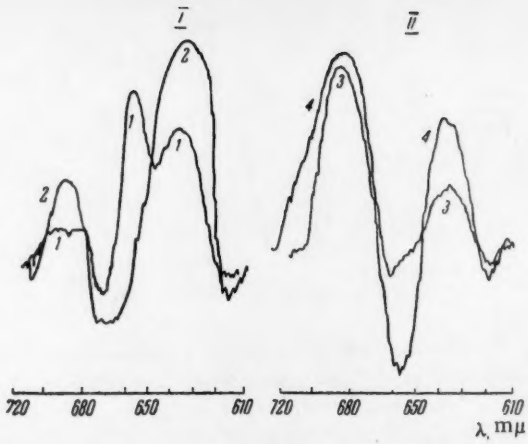

Fig. 9. Effect of pyridine on the fluorescence of unilluminated homogenate (I) and of illuminated homogenate with a fluorescence maximum at 684 mμ (II). 1) Initial unilluminated homogenate, before treatment with pyridine; 2) unilluminated homogenate, containing 2.5% of pyridine; 3) homogenate after illumination for 10 sec; 4) illuminated (10 sec) homogenate, containing 2.5% of pyridine.

684 mμ, by illumination for 10 sec at pH 8.5, and a 680 mμ, by illuminating the homogenate for 30 sec at pH 8.5. The maxima at 684 and 688 mμ were both displaced toward the short wavelength region (to 680 mμ) by thermal treatment or by addition of pyridine; the general level of intensity of fluorescence rose, and the position of the short wavelength maximum remained unchanged at 635 mμ (Fig. 9). These findings are evidence that the effect of the factors examined was to promote transformation of the spectra to that characteristic of chlorophyll and protochlorophyll.

Thermal treatment or addition of pyridine raised the general level of intensity of fluorescence of homogenates which had a fluorescence maximum at 680 mμ after illumination (the final stage of transformation of the pigments), but did not affect the position of the maxima.

DISCUSSION OF RESULTS

The fluoresence spectra of homogenates of etiolated leaves resemble those of intact living leaves [2]. The most intense maximum of the fluorescence spectrum of the homogenate is at 655 mμ, and is due to the presence of a particularly active form of protochlorophyll. Factors which tend to disturb the native structure of the pigment (heat, action of solvents) disrupt the linkage between protochlorophyll and its protein component, as a result of which the pigment undergoes transformation into a more stable form (635 mμ). This transformation is associated with disappearance of the fluorescence maximum at 655 mμ, and with considerable intensification of the maximum at 635 mμ. At the same time, a new maximum appears at 696-698 mμ.

The maxima at 655 and 635 mμ are thus due to different forms of protochlorophyll. The possibility that chemical differences may exist between the different forms of the pigment needs to be investigated; the effects of thermal treatment and of solvents are, however, more suggestive of differences in the form of the linkages of the pigment-protein complex.

A number of workers have, however, demonstrated the existence of intermediate forms of pigment, which differ in the presence or absence of phytol in the molecule [3-6]. Protochlorophyll appears to exist in two equilibrium forms; the form with a fluorescence maximum at 655 mμ is readily transformed into the 635 mμ form, but the conditions under which the reverse process proceeds are not known. The unstable form with a fluorescence maximum at 655 mμ shows very high photobiochemical activity, and is converted completely into the other form when exposed to light. The stable form of protochlorophyll giving maximum fluorescence at 635 mμ is relatively inert, and under our experimental conditions only part of it enters into the series of conversions leading to the formation of chlorophyll. It is possible that the process of chlorophyll formation also proceeds through the 655 mμ form. A large fraction of the 635 mμ form undergoes photooxidation and bleaching during illumination under aerobic conditions.

In contrast to these findings, the form of protochlorophyll giving maximum flourescence at 635 mμ in intact leaves is more active, and practically all of it is converted into chlorophyll. For this reason, all the protochlorophyll present in intact leaves undergoes conversion into chlorophyll. In homogenates, in which the structure of the leaf has been broken down, and in which the state of the pigments has been somewhat modified as a result of this, not all of the protochlorophyll present is converted into chlorophyll.

Formation of the form giving maximum fluorescence at 688 mμ results from the first stage of the series of transformations of pigments in homogenates. This form of chlorophyll appears to resemble that formed in living, uninjured leaves in the initial, photochemical stage of the process, having a fluorescence maximum at 690 mμ [2], and an absorption maximum at 684 mμ [1]. In distinction from intact leaves, however, in which the duration of the first stage of the process is fairly long (up to 10 min), this state is rapidly completed (up to 1 min of illumination) in homogenates, in which the coordination of all the biochemical processes is disturbed to some extent, and the 688 mμ form is quickly transformed into the succeeding form of the sequence of changes.

The processes of conversion of this primary form are strongly inhibited in homogenates containing glycerol, and for this reason only the form showing maximum fluorescence at 688 mμ is found, even after very long periods of illumination (more than 20 min). In homogenates not containing glycerol the 688 mμ form is converted into that giving maximum fluorescence at 680 mμ, the formation of which was established by Krasnovskii and Kosobutskaya [10,11] from the absorption spectra of homogenates. The intermediate atages of the pigment transformations escaped notice in this earlier work, because of the inadequancy of the precautions taken to ensure the total absence of light during preparation of the homogenates, and because of the lower sensitivity of the method of absorption spectroscopy.

Further synthesis of protochlorophyll, and accumulation of chlorophyll, take place in living, structurally intact leaves, during the course of which the linkages between the components of the pigment-protein complexes undergo modification, and the size of the complex increases, as is shown by the shift in the absorption maximum, from 670 mμ to 678 mμ [10, 11]. In homogenates, on the other hand, the series of pigment transformations terminates with formation of primary chlorophyll, with maximum fluorescence at 680 mμ.

An important finding was that factors which disturb the native structure of the complexes (a consequence of which is the disappearance of the form of protochlorophyll which has a fluorescence maximum at 655 mμ), also totally inhibit the ability of the pigment system to respond to illumination. Milder treatment, not causing total disappearance of the form of protochlorophyll fluorescing at 655 mμ, does not abolish the capacity of the pigment system to undergo transformations when illuminated. In such homogenates, which have not been fully denature, the process of chlorophyll formation does not go on to the final state, as in homogenates which have not suffered denaturation, but terminates with formation of intermediate pigment forms, fluorescing at 684 mμ (in the presence of 10-20% of alcohol), or at 682 mμ (after heating at 40°, or in the presence of 1% of pyridine).

The intermediate pigment forms with fluorescence maxima at 684 mμ and at 682 mμ, as well as the initially formed pigment with a fluorescence maximum at 688 mμ, and the chlorophyll with a maximum at 680 mμ, formed as the final product of the series of pigment transformations, all appear to constitute variants of the natural chlorophyll-protein complex. The conclusion is supported by the finding that only residual unconverted protochlorophyll with a fluorescence maximum at 635 mμ, and chlorophyll with a fluorescence maximum at 680 mμ (the stable "monomeric" form of chlorophyll) are found in homogenates containing the initial and the intermediate pigment forms (fluorescence maxima at 688 and 684-682 mμ, respectively), after heat or solvent treatment. The presence in homogenates of etiolated leaves of the pigment form with a fluorescence maximum at 655 mμ is a necessary condition for the process of pigment transformation initiated by illumination.

Further researches should be directed toward the elucidation of the nature of the linkage between protochlorophyll and protein, on which depends the reduction of "semiisolated" double bonds in the molecule of the former substance. In distinction from homogenates, un-

specific photoreduction of dissolved protochlorophyll leads, as has been shown earlier [18], to reversible reduction in the system of conjugated double bonds in the cycle.

SUMMARY

Two forms of protochlorophyll are present in homogenates of etiolated bean leaves, as well as in the intact leaves. One form, with a fluorescence maximum at 655 mμ, is very labile, and exhibits high photochemical activity, and is transformed quantitatively into chlorophyll when exposed to light. The other form, with a fluorescence maximum at 635 mμ, is much less active, and when illuminated only part of it goes to chlorophyll while most of it undergoes bleaching.

Fluorescence spectroscopy at the temperature of liquid nitrogen allowed the sequence of pigment transformations taking place during illumination of homogenates to be followed. The first, rapid state of the process involves formation of a pigment with a fluorescence maximum at 688 mμ. This primary form of chlorophyll then undergoes further transformations, terminating in the production of chlorophyll with a fluorescence maximum at 680 mμ. This series of transformations taking place in illuminated homogenates can be accomplished over a wide pH range. In distinction from intact leaves, further illumination of homogenates does not lead to a further shift in the fluorescence maximum, to 686 mμ.

In the presence of 60% of glycerol in the homogenates the process is arrested at the stage of formation of chlorophyll with a fluorescence maximum at 688 mμ. The active form of protochlorophyll fluorescing at 655 mμ is completely denatured by the action of heat or of solvents on the homogenates, in which chlorophyll formation can then no longer proceed.

The authors wish to express their profound gratitude to F. F. Litvin for his help in interpreting the results of this research, and in using the fluorescence spectrophotometer.

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SCIENTIFIC INFORMATION

PROBLEMS OF MIGRATION OF ENERGY AND OF ELECTRONS IN PHOTOSYNTHESIS AND OXIDATION REACTIONS

(From the General Discussion at the Faraday Society Meeting, April 14-16, 1959)

Translated from Biokhimiya Vol. 25, No. 1, pp. 180-186, January-February, 1960

The Faraday Society held one of its periodical discussion meetings at Nottingham University (England) in April 1959, on the subject of "Energy transfer with special reference to biological systems".

The discussion program comprised two sections: I) "Modes of energy transfer from excited and unstable ionized systems" (10 papers); II) "Energy migration in organized biological systems" (16 papers). Section I, dealing with transfer of excitation energy in solutions of fluorescent organic substances and in luminescent organic crystals, excited by ultraviolet light or γ-radiation, will not be considered in the present review.*

In this review we shall give an account of the papers dealing with photosynthesis and with enzymic oxidation-reduction processes. Many of the papers were presented in so condensed a form as to necessitate a more systematic and informative presentation in this review.

Prof. Livingston, known for his work on the photochemistry and luminescence of chlorophyll in solutions, read a joint paper with Pugh, of the University of Minnesota, entitled: "The role of the triplet state in reactions sensitized by chlorophyll". The authors first expressed their views on the mechanism whereby the energy of excitation of chlorophyll is utilized, and then presented the results of their research on a particular apsect, viz., formation of intermolecular associations of chlorophyll and retinene in solutions.

In accordance with the viewpoint also consistently developed by us, these authors believe that the triplet state of the chlorophyll molecule is an essential intermediate in its photochemical reactions in homogeneous solutions. These authors consider that this conclusion can be derived unequivocally from comparisons of the initial quantum yield of fluorescence, due to the singlet excited state, with the photochemical yield of the reaction, when the reagents are added to the fluorescing solution of chlorophyll: the yield of the reaction considerably exceeds the yield of fluorescence of the solution before addition of the reagents. Apart from this, the flash technique, which permits of the accumulation of large amounts of chlorophyll in the triplet state, and of direct spectroscopic measurement of the kinetics of decay of such molecules, also confirms the importance of their role in photochemical reactions taking place in solutions. However, a similar experiment performed by other workers, using chlorophyll suspensions instead of solutions, did not reveal any significant amounts of triplet molecules, evidently because of their greatly shortened mean life in vivo. The authors believe that concentrational inactivation of triplet molecules of chlorophyll by normal ones, i.e., self-quenching of the triplet state, is negligibly small at concentrations such as prevail in chloroplasts (of the order of 0.1 M).

It is quite surprising that the fluorescent, excited state of chlorophyll is also not subject to any appreciable self-quenching in chloroplasts. The authors came to this conclusion on the basis of their calculations of maximum quantum yield of fluorescence. Self-quenching of the fluorescence of molecules when their concentration is raised is usually ascribed to inductive transfer of energy of excitation between the molecules until an adventitious, nonfluorescent dimeric molecule is encountered. The absence of self-quenching of fluorescence of chlorophyll is considered by these authors to be evidence of the absence from chloroplasts of such dimers, which are able to degrade captured energy of excitation to heat. There can be no doubt as to the existence of energy migration within chloroplasts, since depolarization of fluorescence is about the same as it is in 0.1 M solutions in viscous solvents, as was shown by earlier work.

The authors advanced the following scheme for the process: Energy of excitation is able to migrate freely by an induct ve mechanism of transfer within the condensed system of the chlorophyll molecule, without suffering quenching (probably because of the absence of dimeric molecules from the monomolecular layer), until it reaches a chlorophyll molecule which is in a specially favorable situation for the development of the biochemical reaction of photosynthesis. This molecule then undergoes a transition from its fluorescent to its triplet state, the mean life of which is markedly shortened because of the chain of biochemical reactions initiated. Livingston and Fujimori had earlier investigated the effects of oxidizing and reducing agents on triplet molecules of chlorophyll produced by exposing solutions to powerful light impulses, in order to elucidate the nature of this biochemical process. As was

*For a report on the discussion as a whole see the author's paper in Vestnik Acad. Nauk SSSR, No. 8, 49 (1959). Detailed reports on some of the papers of section II have been submitted by the author to Biofizika (No. 1, 1960).

to be expected, oxidizing agents were found to have a strong reversible quenching effect on the chlorophyll triplets (oxygen, quinone, m-dinitrobenzene, β-carotene), with the virtual absence of irreversible processes. According to the findings of A. A. Krasnovskii, β-carotene is a powerful inhibitor of the reaction of photoreduction of chlorophyll by ascorbic acid, which is in agreement with our hypothesis that the triplet state of the chlorophyll molecule is concerned in this reaction. Livingston and Fujimori found, however, that typical reducing agents (ascorbic acid, allylthiourea, phenylhydrazine) did not, even when present in high concentration (0.1 M), exert any quenching effect on chlorophyll triplets in methanol or pyridine solutions. During the discussion of Livingston's paper, the author of this review drew attention to the inconsistency of this result with the reversible photoreduction of chlorophyll discovered by A. A. Krasnovskii, and studied in detail by V. B. Evstigneev, involving the formation of a primary reduced form (semiquinone) of chlorophyll. These authors showed that the reductants did not quench the fluorescence of chlorophyll, and hence did not react with its excited (singlet) state. Since only two levels of excitation of the chlorophyll molecule are known at present – the singlet and the triplet states – and since neither of them is sensitive to reductants, we are confronted with the paradoxical position of not being able to decide which of the excited forms of chlorophyll is concerned in the photoreduction reaction. For this reason, we had certain reservations regarding the validity of Livingston's assumption that all the absorption bands appearing on exposure of chlorophyll solutions to flash impulses are due to its triplet molecules. We concluded, from an examination of the absorption spectrum of hematoporphyrin solutions following flash illumination, that, of the three maxima observed, only one can be related to the triplet state, while the other two coincide with those of the partly reduced (semiquinone) form of the pigment, as recorded by V. B. Evstigneev. These findings indicate the necessity for revision of existing interpretations of spectral changes appearing in chlorophyll solutions exposed to powerful flash illumination, and also the need to take into account the reducing power of the solvent (pyridine containing water). For chlorophyll, the possibility of its self-reduction by its own phytol tail also exists.

In addition to reviewing published work, Livingston and Pugh's paper reported on new work on the quenching of the triplet state of chlorophyll by retinene – an analog of the carotenes. The effect of retinene on the rate of quenching of the chlorophyll triplet state differed from that of carotenes in that at high concentrations the rate of decay ceased to be a linear function of the retinene concentration, but approached a limiting value. The authors advanced the interpretation that retinene and chlorophyll form a reversibly dissociable addition compound, in which chlorophyll retains its usual absorption spectrum, but the half life of the triplet state of the addition compound is about one sixth of that of free chlorophyll. The authors regarded this finding as affording confirmation of the above hypothesis as to the shortening of the half-life of the triplet state of a chlorophyll molecule situated in proximity to a center of biochemical activity.

The contribution of the three American authors, Lumry, Mayne and Spikes, was entitled: "Fluorescence yield against velocity relationships in the Hill reaction of chloroplast fragments." These authors conducted a detailed parallel study, on the same material, of the dependence of the relative yield of fluorescence of chlorophyll, on the one hand, and of the Hill-reaction velocity (after evolution of oxygen), on the other hand, on the intensity of incident light (of λ 440 mμ), over a thousandfold range of intensities. Chloroplasts were taken from two species of plants. Potassium ferricyanide was used as the oxidant in the Hill reaction. The curves obtained for reaction rate and for fluorescence yield both tended towards a limiting value as the intensity of illumination was increased. The authors expressed the view that chlorophyll molecules are grouped in photosynthetic units, also containing special centers which act as traps of the energy of excitation of chlorophyll, and which release the reaction of production of oxygen from water. This model of a photosynthetic unit is not, however, a necessary consequence of the experimental evidence adduced in this paper, but is taken by the authors as having been established by Wessels and Spikes' (1956) finding that chlorophenyldimethylureas completely eliminate the Hill reaction in concentrations of one molecule of inhibitor for each several hundred chlorophyll molecules.

From a theoretical analysis of their experimental curves the authors proposed the following simple scheme of elementary processes:

$$C \xrightarrow{\alpha I} C^* \quad \text{(a, absorption of light)}$$

$$C^* \begin{cases} \xrightarrow{k_f} C + h\nu & \text{(b, emission of fluorescence)} \\ \xrightarrow{k_h} C + \text{heat} & \text{(c, degradation to heat)} \end{cases}$$

$$C^* + T \xrightarrow{k_t} \{T\} + C \quad \text{(d, energy trapping)}$$

where C * is a chlorophyll molecule in its ordinary (singlet) excited state, and T is an energy-trapping complex, converted by the trapped energy into the reactive from $\{T\}$, which initiates a series of intermediate forms leading to the evolution of oxygen. The superscripts over the arrows indicate that the reaction rates of the individual processes are determined by the constants k_f, k_h, and k_t, and that the primary process of excitation is proportional to the incident light intensity I. In conformity with the generally known phenomenon of light saturation, it is supposed that the complex T is open to accept energy transmitted to it from chlorophyll over a period of 10^{-2} sec, after which it closes, so that if energy quanta are supplied to it at shorter intervals it cannot accept them. The constant k_t measures, relatively to k_f and k_h, the probability that a quantum of excitation energy, the life-

time of which is of the order of 10^{-9} sec in chlorophyll, will in its migration within a photosynthetic unit get to T, and be accepted by it. A detailed mathematical analysis of this scheme, taking into account the dependence of the velocity of the Hill reaction of illumination received during the experiment, and using the reaction curve parameters, made it possible to construct a curve relating fluorescence yield to light intensity I. The authors considered that this provides full confirmation of the validity of the above scheme, in which the rate-limiting steps are the degradation process (c), and the trapping of an energy quantum by complex T (d).

From the satisfactory agreement found with the above scheme, which envisages only three alternatives, viz, fluorescence, degradation, and energy trapping, the authors concur with Livingston (preceding paper), in concluding that ordinary self-quenching of fluorescence, such as is found in concentrated solutions, does not occur in a photosynthetic unit made up of chlorophyll molecules, and that this difference is ascribable to some special structural organization of the chloroplasts. The authors reject the possibility of the existence of a semiconductor mechanism for the migration of energy quanta within a photosynthetic unit.

E. Rabinowitch read a paper entitled: " Primary photochemical and photophysical processes in photosynthesis." His paper covered the following subjects: 1) Excitons and their migration; 2) structure of chloroplasts; 3) states of chlorophyll in the chloroplasts; 4) energy and charge transfer in chloroplasts; 5) photochemical function of chlorophyll.

In the first part of this paper the author gave a graphic account (for the benefit of biologists present in the audience) of the three known types of energy migration which may be encountered in a system made up of identical molecules (as, for example, in a crystal lattice): (A) Transfer and migration of an intramolecular exciton from one molecule to a neighboring one, by an inductive mechanism, which was discussed above; (B) transfer and migration of an intramolecular exciton between neighboring molecules, superficially identical with A, but involving simultaneous exchange of electrons in opposite directions, viz., between a vacant ground level of an excited molecule and a filled ground level of a nearby molecule, and between a filled excited level of one molecule with a vacant one of the other; (C) complete dissociation of an excited electron from a molecule, with its subsequent migration, together with the hole, or independently of it, through the molecular system, with development of primary conductivity in the former case, or of secondary conductivity in the latter. Usually only transfer types A and B are considered in relation to migration of excitation energy in the condensed chlorophyll system present in chloroplasts. Mechanism C was considered in our paper only.

Rabinowtich concludes, from the electron-microscope data a number of workers, showing the lamellar structure of grana and chloroplasts, that chlorophyll is present as a monomolecular layer, with a random alignment of molecular axes. The latter conclusion was based on the experiments of Jacobs and Rabinowitch on the absorption spectra of artificially prepared monolayers of chlorophyll; these may be obtained either as amorphous or as crystalline aggregates. For the latter, an absorption maximum was found 735 mμ (740 mμ for chlorophyll crystals), showing a considerable shift in the absorption band peak (660 mμ). The absorption maximum for amorphous chlorophyll monolayers was at 678 mμ. According to Rabinowhich, this finding constitutes convincing evidence that the chlorophyll present in living cells, which has an absorption peak at 675-680 mμ, cannot be in a crystalline form, or, at least, that it cannot be in the form of extended crystals, as was postulated by Calvin for the purposes of his semiconductor theory of photosynthesis. Arnold's experiments, which showed the presence of free electrons in dried chloroplasts, cannot, in Rabinowitch's opinion, be considered to apply to the conditions prevailing in living chloroplasts.

In order to reconcile discordant in vivo measurements of lifetime of fluorescence, on the one hand, and of the quantum yield of fluorescence, on the other, Rabinowitch supposes that only a quarter of the chlorophyll present in Chlorella is able to fluoresce, with a yield of 10%, while three quarters is in a nonfluorescing form. Such proportions would then give the observed average yield of 3%. The hypothesis had been advanced earlier by A. A. Krasnovskii that at least two forms of chlorophyll are present in living leaves: a fluorescent, monodisperse form, and a nonfluorescent, aggregated form. Similar views are now being developed by G. Franck.

Rabinowitch supported the concept of photosynthetic units, each served by a definite number of chlorophyll molecules, but he reduced their number from 2000 to the more reasonable figure of 250, based on the grounds that liberation of one molecule of O_2 from water requires not one, but probably 8 identical elementary photochemical processes. Rabinowitch gave a graphic representation of such a unit, as an amorphous monolayer of 200 chlorophyll molecules, enclosed in a protein envelope, this being a macromolecule of the enzyme responsible for the biocatalytic reaction. Calculation showed that during the lifetime of the excited state, of the order of 10^{-9} sec, a quantum of excitation energy may visit up to 1000 chlorophyll molecules, staying in each for at least 10^{-13} sec. This last requirement emerges from the observation that the absorption spectrum of chlorophyll shows marked vibrational characteristics, and hence that resonance with the intramolecular vibrations must require a duration of the excitation state not shorter than the period of vibration, of the order of 10^{-13} sec. Rabinowitch did not favor the hypothesis of migration of excitation energy at the triplet level of the pigment, which was discussed by the author of this review in his paper, and which awaits additional quantitative confirmation. Rabinowitch pronounced

definitely against the semiconductor hypothesis of photosynthesis of Calvin, and considered that the production of magnetic signals due to electron spin, registered in electron resonance experiments, as well as the presence of photoconductivity, are evidence of the existence of side-reactions, of secondary importance, and proceeding with a much lower quantum yield than in photosynthesis. However, he admitted the possibility of migration of charges within the boundaries of the pigment monolayer, and this distinguishes his concept from that of Calvin. Rabinowitch considers that with a monolayer spatial separation of the primary products of the oxidative stage of photosynthesis from the products of the reductive stage is a natural consequence of these stages taking place on opposite surfaces of the chlorophyll monolayer. The need for the migration of charges from the site of oxidation through the bulk of the crystalline chlorophyll to the site of reduction, postulated by Calvin, is obviated by Rabinowitch's scheme, since only the thickness of the monolayer separates these two processes.

Emerging from the work of A. A. Krasnovskii and V. B. Evstigneev, Rabinovich et al. showed, from the difference spectra of Chlorella, that when photosynthesis becomes light-saturated, up to 0.3%, i.e., 1/300th of the total chlorophyll content, undergoes conversion into a secondary (red), photoreduced form of chlorophyll. They hence drew the conclusion that of the 250-300 chlorophyll molecules present in a photosynthetic unit only one undergoes reduction during trapping of excitation energy, evidently because it is situated next to the biocatalytic center.

It thus appears from the papers read by Livingston, Lumry, and Rabinowitch that the concept of photosynthetic units, first advanced 23 years ago by Gaffron and Wohl (1936), has received unexpectedly weighty support from the results of recent experimentation.

To the section dealing with photosynthesis belonged the communication read by the Dutch biophysicists Amesz and Duysens, entitled: "Spectrophotometric studies on pyridine nucleotide in photosynthetic cells and cellular material." Applying optical methods, these authors investigated the photoreduction of DPN and TPN added to suspensions or extracts of spinach chloroplasts, during exposure to red light, which was absorbed by the chlorophyll. They first followed the reactions in living cells and in chloroplast preparations, measuring the blue fluorescence of the reduced forms of pyridine nucleotides. They found, however, that these measurements involved a considerable, unassessable error, due to combination of reduced pyridine nucletide with some cell constituent, with resulting enhancement of its fluorescence, as compared with that of the free nucleotide. They therefore used an absorption method, depending on change in optical density at 350 mμ, at which reduction of pyridine nucleotides causes a considerable shift. Such measurements showed that the quantum yield of photoreduction in chloroplast suspensions amounted to 6 quanta (680 mμ) per TPN molecule. Since the free energy change of over-all reaction of hydrogen transfer: $TPN + H_2O \rightarrow TPN\text{-}H + {}^1/_2O_2$ amounts to 52 kcal/mol, and the energy of 6 quanta to 252 kcal, utilization of incident light energy would amount to 21%. If all of the reduced pyridine nucleotide formed were used for reduction of CO_2 in the photosynthetic process, then, from this value for the quantum yield of photoreduction of pyridine nucleotides, a quantum yield (requirement) of 12 quanta per O_2 molecule would follow for the photosynthetic process; this is much closer to Emerson's value of 8-10 quanta than to Warburg's (2.8-5 quanta).

From photometric measurements of the dependence of the velocity of reduction on the concentrations of DPN and TPN added to the chloroplast preparations, and on the light intensity (680 mμ), the authors conclude that it is chiefly TPN that is reduced, and that both TPN and TPN-H strongly inhibit photoreduction of DPN. This would rule out the possibility of the transhydrogenation reaction: $TPN\text{-}H + DPN \rightleftharpoons TPN + DPN\text{-}H$. It was not possible, from the available kinetic data, to decide whether a single enzyme catalyzes the reduction of both nucleotides, or whether two different enzymes are involved.

Another problem raised in the biological section of the discussion was that of electron transfer along the chain of respirator enzymes. A highly condensed paper by Chance and Spencer was devoted to this subject. Using a very sensitive difference absorption spectrum method, these authors found that oxidation-reduction processes taking place at the temperature of liquid nitrogen (−195°) in the cytochrome chain of yeast cells involve only partial, but not complete, reduction of all the cytochromes present. With their spectrometric equipment, and at the low temperature taken, the authors were able to measure accurately the relative concentrations of the reduced forms of cytochromes b, c_1, and c separately, since the maxima of the absorption spectra of the reduced cytochromes (falling within the range 559-547 mμ) were distinct, without any overlapping. The apparatus, described in an earlier publication, serves for automatic, high-speed recording of absorption differences between two cuvettes containing yeast suspensions in buffer solutions, to one of which been added ethanol as a substrate of aerobic oxidation. Glucose was taken in some cases as an alternative substrate. Comparison with the spectra of cytochromes fully reduced at 20° showed that at low temperatures, in the steady state, only 65% of cytochrome b, 8% of cytochrome c, and 27% of cytochrome c_1 were present in the reduced form. It may hence be concluded that transfer of electrons along the chain of oxidized and reduced forms of cytochromes is slowed down, or even totally arrested, at the temperature of liquid nitrogen. Transfer of electrons proceeds rapidly, however, at room temperature. These results bear directly on the question of whether electron transfer in the cytochrome system occurs through immobile or mobile carriers. Since glycerol exerts a considerable inhibitory effect on electron transfer, without significantly affecting the relative steady-state concentrations

of the reduced cytochromes, this is considered by these authors to support the mobile carrier idea. It does not support the hypothesis of immobilized carriers in which electron transfer occurs through conduction bands or coupled electromagnetic oscillations. These authors do not consider, however, that the available experimental evidence is sufficiently conclusive, and they intend to continue their researches on the subject.

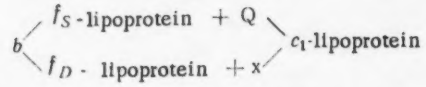
The paper presented by Williams, Cowan and Drake was entitled: "Models for some energy-transporting substances of biological interest." The model systems examined included ferro- and ferricomplexes of dimethylglyoxime (DMG), of the composition $Fe^{n}(DMG)_2X_2$, where X = H_2, pyridine, ammonia, CN^-, OH^-, CO, O_2, and other monodentate ligands. The authors compared the properties of these complexes with those of the analogous ferro- and ferriporphyrin complexes. Comparison of the magnitudes of the magnetic moments, and of the postions of the absorption spectrum maxima (in the 600-500 mμ range), of the members of the DMG and the porphyrin systems, depending on the nature of the ligand, permitted of the elucidation of the electron bonding in these complexes, and of a rational explanation of the opposite direction of the shifts in the absorption spectrum maxima in the two series, with transition from one ligand to another. The authors believe that in the complexes $Fe^{II}(DMG)_2X_2$ the nature of the absorption spectrum depends basically on the transition of the 3d electron of Fe to a higher level (4p) of the same center, although there must also be considerable coupling with the ligand (DMG) levels. On the other hand, in the porphyrin complexes $Fe^{II}PX_2$ (P represents the porphyrin ring system) absorption of a quantum of light energy leads to excitation, not of the Fe electron, but of one of the electrons of a pyrrole ring nitrogen atom, forming donor bonds to the Fe^{II} cation. Excitation of this electron causes its transition to the π-orbitals of porphyrin. This difference in the origin of the spectral bands of the two classes of complexes explains qualitatively the opposite direction of the shifts in the position of the absorption maxima when one ligand X is substituted for another. Transition of a Fe^{II} electron to the 4p level, coupled to the π-orbital of porphyrin, is analogous to that observed in DMG complexes, and can also occur in this case, but the corresponding absorption spectrum maximum is low, and is masked by the absorption of porphyrin itself. A second absorption maximum is present in the spectrum of pyridine-DMG complex, in the 400-500 mμ band, and this is ascribed to a 3d to 4p excitation of the ferrous electrons, coupled with the π-orbital of pyridine. This maximum is absent when the ligand is imidazole. The authors draw the conclusion, of importance for the biological process of electron transfer, that electron migration in porphyrin complexes, bound through Fe^{II} to imidazole, probably proceeds through the porphyrin ring, rather than through the imidazole ligands. Presence of ligands such as CO or CN^- inhibits iron porphyrins by blocking migration of the electrons in the porphyrin ring.

An examination of the spectra of the ferriporphyrin complexes $Fe^{III}PX_2$ led to the rejection of Williams' earlier attribution of the bands at 475 mμ and 625 mμ to transition of porphyrin electrons to the Fe^{III} cation. They are due to the complexed Fe^{III} cation itself, since they are also encountered in DMG complexes, which have quite different electronic structure and orbits.

The authors showed that a linear relationship exists between the positions of the absorption maxima of complexes of the series $Fe^{II}(DMG)_2X_2$ and of the series $Fe^{II}PX_2$, different porphyrins giving parallel lines on the graph. This relationship makes it possible, from the positions of the absorption maxima of different hemoproteins, such as hemoglobin and the cytochromes, to draw conclusions regarding the protein groups (imidazolyl, amino, carboxyl) bound to the heme. This linearity rule was found to apply only to complexes of sterically regular structure, so that qualitative conclusions could be made as to the degree of maladjustment of ligands around the Fe cation from the magnitude of the deviation from linearity of the curves for a given complex with different X groups. Thus the maladjustment in iron porphyrin protein bound through imidazole diminished in the sequence myoglobin, hemoglobin and chlorocruorin, cytochrome a_2, cytochrome a_3, cytochrome a, cytochrome c. Maladjustment around the Fe is slight in cytochrome b, where Fe is bound through amino groups. In peroxidases and catalases heme Fe is bound to carboxyl groups. Finally, the authors presented their data for the equilibrium constants for formation of the complexes $Fe^{II}(DMG)_2XY$, where X is hydrazine, and Y is pyridine, imidazole, histidine, CN^-, and other ligands. Of the nitrogen bases, imidazole was the most firmly bound. The diimidazolyl complex was able to take up oxygen reversibly, as was shown by spectrophotometric observations.

D. Green's scheduled paper was not delivered; instead, a paper was presented on the subject: "Structure-function interrelationships in mitochondrial electron transport and oxidative phosphorylation." From electron microscope examinations of mitochondria, and from the results of his own experiments on their fragmentation, the author concludes that it is possible to prepare mitochondrial fragments possessing an intact electron transfer chain from substrate to molecular oxygen, but which are not able to carry out the Krebs citric acid cycle, and do not have the capacity for oxidative phosphorylation. Other fragments (mitochondrial particles) have an intact electron transfer chain, and have the capacity for oxidative phosphorylation, but they cannot carry out the Krebs cycle. Such particles still retain the double layer structure. The author then proceeded to discuss only fragments with an intact electron transfer chain; by further fragmentation of such particles, with such reagents as cholates, alcohols, and detergents, with examination of the specific enzymatic activities of the resulting products, he attempted to reconstruct the order in which the enzymes act in the original intact mitochondrion. From such a reconstruction he

postulates the following scheme of interrelationships of the intramitochondrial electron-transporting enzymes, viz., two flavoproteins (succinic dehydrogenase, f_S, and DPNH dehydrogenase, f_D), four cytochromes, a, b, c_1, and c, and coenzyme Q:



The author considered that the electron-transferring coenzyme Q (of a quinonoid structure) is present in the lipoprotein separating f_S from c_1. An analogous, as yet unidentified, component x serves as an electron carrier between f_D and c_1, also through a lipoprotein phase.

A short paper by Gutfreund, entitled: "The transfer potential of enzyme-substrate compounds," concluded the series of papers read in the biological section. The author restricted himself to a general thermodynamic consideration of the known problem of intermediate compounds of enzymes with substrates, as factors determining the basic mechanism of enzyme action. As a result of combination of the substrate with the enzyme, the free energy of activation of the intermolecular transfer of groups of atoms is lowered to that of addition of the substrate to the enzyme. It is regrettable that the author did not enter into a detailed discussion of the nature of the reactive groups and catalytic centers at the enzyme surface, although this subject has been extensively discussed in the literature, but made only passing references to it. Of the work of his own laboratory, he mentioned only a presumptive conclusion that in chymotrypsin the serine OH-group is activated by formation of a hydrogen bond with histidine, while in ficin activation is due to a similar interaction between a carboxyl and a sulfhydryl group.

Gutfreund considers that the detailed examination of enzyme-substrate intermediates should render superfluous the vague concepts of one reaction driving another. He sees no basis for concepts involving energy transfer at a distance, and considers that the basic mechanism of the usual biochemical processes involves interaction and transfer of groups of atoms, proceeding at an enzyme surface. Although this author did not once mention free radicals, his concept of regrouping of molecules at enzyme-catalyst surfaces must, when developed with greater precision, lead to that of free radical reactions.

Gutfreund was fully justified in objecting to the term "high energy phosphate bond," which has received wide acceptance in biochemical terminology. He objects to the commonly used statement that "the phosphate bond energy of ATP is 8000 cal/mole," since it refers not to bond energy as this term is understood in chemistry, but to the free energy of hydrolysis of the reaction: ATP + $+ H_2O \rightleftharpoons$ ADP + P_{inorg}. In order to avoid such ambiguities, the author proposes the introduction for a range of acyl compounds of a scale of free-energy changes during hydrolysis, and of the general term "group transfer potential", of which hydrolysis would represent a special case, with water as the acceptor.

It should, in conclusion, be remarked at the Faraday Society Discussion embraced too wide a range of phenomena of energy transfer, from physical inductive transfer of energy of excitation between molecules to electron transfer along a chain of cytochromes in a lipoprotein phase, as in mitochondria. This overwide range of subjects did not permit of the organization of fruitful discussion of concrete cases belonging to the borderline between biochemistry and physical chemistry. For this reason the majority of the contributors confined themselves to the communication of new, original work, or to comparatively superficial comments on the program papers, expressing their own views on the given subjects. The discussion would, from this point of view, have benefited considerably from the omission of the first, purely physical section from the program, so allowing more attention to be devoted to the physicochemical problems of biochemistry.

Nevertheless, personal contact between scientists working in different specialties undoubtedly helped to promote the inception of new ideas and approaches in the fascinating borderland between chemical physics and biochemistry.

A noteworthy omission from the proceedings was that no mention was made of the applications of the methods of paramagnetic electron and nuclear resonance to the study of biological problems, although such methods are now being intensively developed for this purpose. We think that this constitutes a serious omission from the discussion.

A. N. Terenin

BIOCHEMIST NOBEL PRIZE WINNERS OF 1959

LABORATORY SYNTHESIS OF NUCLEIC ACIDS USING ENZYMES

Translated from Biokhimiya Vol. 25, No. 1, pp. 187-189, January-February, 1960

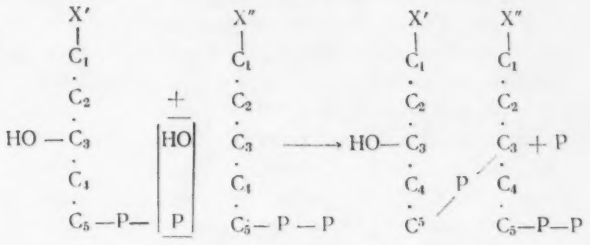
Important advances have been made within the past ten years in the study of the biochemistry of the nucleic acids. First of all, the chemical structure of the nucleic acids has been established. It has been shown that both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) are high-molecular polymers built up from a large number of mononucleotide units – the purine and pyrimidine nucleotides. It has been shown that the nucleotide units of both RNA and DNA are linked through phosphoric acid residues, connecting the 3'-hydroxyl of the carbohydrate residue of one nucleoside to the 5'-phosphodiesteratic linkages. The polynucleotides so formed (whether ribose or deoxyribose) are invariably unbranched chains, which may attain a very high degree of polymerization, with the chain consisting of 6000 or more mononucleotide residues. The complete DNA molecule is made up to two such polymeric chains coiled around each other, and bound together by a rigid system of hydrogen bonds.

The most interesting property of nucleic acids, discovered only within the past few years, is their specificity. It was found that nucleic acids of different biological origin contained different relative proportions of their constitutent nucleotides (adenylic, guanylic, cytidylic, and uridylic acids in RNA, and deoxyadenylic, deoxyguanylic, desoxycytidylic, methylcytidylic, and thymidylic acids in DNA), and that they also differed in the order in which these nucleotides were arranged along the chain. The relative proportions of the nucleotides (nucleotide composition) present in nucleic acids are specific for a given species, and are in close relation with the hereditary characteristics of organisms. The specificity of nucleic acids, and their relation to heredity, were also revealed in experiments on transformation of bacterial types by the action of DNA, and on the infectiveness of virus RNA, which showed that nucleic acids were carriers of definite, narrowly specific, heritable characteristics.

There is also a considerable amount of evidence that nucleic acids, in particular RNA, participate in the process of biosynthesis of protein. It has been found that nucleic acids play a dominating role in this vital process. The contributions of numerous workers have led to the idea that the specific arrangement of amino acids in the polypeptide chain of proteins is determined by that of the nucleotides in the polynucleotide chain of nucleic acid, which thus serve as templates for reproducing the structure of proteins.

It is obvious that the mechanism of the biological or laboratory synthesis of such biologically important substances as are the nucleic acids must be a problem of the first importance to biochemists. Up to 1955, however, nothing definite was known about the mechanism of formation of polymeric RNA or DNA chains from nucleotides, and there was not even any clear indication of an approach to the investigation of this problem.

In 1955 the well-known American biochemist Severo Ochoa announced, in a joint paper with the French scientist Marianne Grunberg-Manago, that they had isolated a new enzyme from bacterial cells, which could under ordinary laboratory conditions catalyze the polymerization of monoribonucleotides, with the production of high-polymeric polyribonucleotides of the RNA type. This reaction would proceed only when the monoribonucleotides were present as nucleoside-5'-diphosphates. One phosphate residue was eliminated during condensation, and to the remaining phosphate residue at position 5' of the ribose molecule was joined the 3' hydroxyl of the ribose component of a second nucleotide:



The nucleotides of the polymer formed in the presence of this enzyme were thus joined by 3', 5'-phosphodiesteratic linkages, which are present in all the naturally occurring nucleic acids. It followed that the products of enzymatic polymerization obtained under laboratory conditions did not differ from natural RNA in this respect.

When these synthetic polyribonucleotides, or any natural RNA, were incubated with the enzyme in the presence of inorganic phosphate, depolymerization took place, with addition of inorganic phosphate to that at position 5' of the ribose unit, giving nucleotide-5'-diphosphate. In other words, the enzyme acted reversibly, the reverse reaction involving the enzymatic phosphorolysis of the phosphodiesteratic 3', 5'-linkages of RNA. For this reason, the enzyme was named polynucleotide phosphorylase, because it catalyzed both the polymerization of phosphorylated nucleotides with production of an ordinary polynucleotide chain, and the reverse reaction of phosphorolysis of polynucleotide with production of free phos-

phorylated nucleotides. The reactions may be represented in the following schematic form:

$$n\,X{-}R{-}P{-}P{-}P \rightleftarrows (x{-}R{-}P)_n + nP,$$

where R is a ribose residue, P is phosphate, and X is one of the nitrogen bases of RNA (adenine, guanine, cytosine, or uracil). The enzyme was found to be widely distributed among the bacteria, and has also been found in some animal and plant tissues. The wide distribution of polynucleotide phosphorylase in the organic world suggests that it may also be implicated in the intracellular synthesis of RNA

The discovery of the possibility of enzymatic synthesis of polyribonucleotides in the laboratory inaugurated a whole series of the most diverse research projects by various workers, dealing with the study of the polymers produced by this reaction. The discovery opened up a new chapter in nucleic acid chemistry - the chemistry of the artificial polynucleotides.

The study of the artificially synthesized nucleic acids proceeded rapidly, and it now constitutes a whole separate branch of research work. It has been shown, using various methods, that the micromolecular structure of artificial polyribonucleotides made up of the four types of nucleotide residues (adenylic, guanylic, cytidylic, and uridylic acids) is identical with that of natural RNA. If, moreover, the reaction mixture contains all four nucleotides in equimolar proportions, the nucleotide composition of the resulting polymer closely resembles those of naturally occurring ribonucleic acids from bacteria and other organisms. Synthetic ribonucleic acids have been prepared with a molecular weight of up to 2 million, which also corresponds to maximum values found for naturally occurring RNA. X-ray studies have shown that the diffraction diagrams of natural and synthetic polyribonucleotides are identical. All these findings show that polyribonucleotides can in fact be synthesized enzymatically in the laboratory, and they point to a possible pathway of enzymatic synthesis of RNA within living cells.

These researches have not, however, thrown much light on the problem of the specificity of the biosynthesis of natural RNA. Polynucleotide polyphorylase was shown to be an enzyme which could promote the synthesis of polynucleotides of any nucleotide composition, depending on the nature and relative proportions of the components introduced into the reaction mixture; the order of incorporation of these components must, however, have been a random one. The discovery of this enzyme has not, therefore, contributed to the solution of the problem of what cell mechanisms are concerned in ensuring a specific arrangement of the nucleotide residues along the chain of natural ribonucleic acids. Certain experimental data have, however, recently been obtained in S. Ochoa's laboratory, bearing on this problem. It was shown that the nature of the synthesized polyribonucleotide depended to a considerable extent on the nature of a primer added to the reaction mixture, in the form of a small amount of any naturally occurring RNA; the nucleotide composition of the enzymatically synthesized RNA was found to resemble the specific nucleotide composition of the natural RNA added as a primer. S. Ochoa is now inclined to interpret his earlier finding that the nucleotide composition of his synthetic RNA resembled that of bacterial RNA as being attributable to the presence of a small admixture of microbial RNA in his enzyme preparations, and that this admixture might have fulfilled the role of a specific primer, ensuring the synthesis of RNA molecules possessing its own specificity. He believes that the primer molecules may fulfil the role of specific templates during the course of enzymatic polymerization of monomers to polynucleotides.

In 1957, two year after Ochoa's discovery, a second American biochemist, Arthur Kornberg, reported the isolation of a new enzyme - polymerase - which catalyzed the synthesis of polynucleotides of the type of DNA. The action of this enzyme differed from that of polynucleotide phosphorylase. It was active with deoxyribonucleotides only. The polymerization reaction proceeded only with phosphorylated deoxyribonucleotides, but, for the action of this enzyme, the nucleoside-5'-triphosphates had to be present, instead of the 5'-diphosphates required for the action of polynucleotide phosphorylase. The reaction catalyzed by polymerase involved splitting off from the phosphorylated deoxyribonucleotides of two phosphate residue at a time (as pyrophosphate), followed by condensation of the remaining 5'-phosphate residue with the 3'-hydroxyl of the deoxyribose component of a second nucleotide. The component units of the resulting polymer were thus linked together by 3',5'-phosphodiesteratic bonds, as in the naturally occurring DNA. The reaction of enzymatic synthesis of polydeoxyribonucleotides may be represented as follows:

$$n\,X{-}D{-}P{-}P{-}P \rightleftarrows (X{-}D{-}P)\,n + nP{-}P,$$

where D is deoxyribose, P is phosphate, and X is any of the four nitrogen bases of DNA (adenine, guanine, cytosine, or thymine). The polydeoxyribonucleotides thus synthesized by an enzymatic reaction in the laboratory did not differ in their physical and physicochemical properties from naturally occurring native DNA, suggesting that they had a common macromolecular structure.

It was shown in the first papers of A. Kornberg and his collaborators that the presence of a small amount of high-polymer DNA from natural sources, acting as a primer, was essential for the enzymatic synthesis of artificial DNA. They also showed that the nucleotide composition of the resulting synthetic DNA was identical with that of the DNA taken as the primer. Thus, for example, when they added primer DNA with a higher content of adenine + thymine than of guanine + cytosine (an AT-type of DNA, such as that prepared from pneumococci or from animal tissues), the polymer synthesized was also of the AT-type. If, as the primer, they took a GC-type DNA (containing more guanine + cytosine than adenine + thymine, such as the DNA from Mycobacterium tuberculosis), the resulting poly-

deoxyribonucleotide was also of the GC-type. Thus the DNA synthesized in the laboratory always had the same specific nucleotide composition as did the DNA taken as the primer.

This discovery was of the utmost importance, since it led to the elucidation not only of the pathway of enzymatic biosynthesis of DNA from low-molecular weight precursors, but also of the mechanism whereby the narrow specificity of nucleotide composition and sequence of DNA may be achieved in the cell. These experiments of Kornberg and his associates have provided a definite experimental basis for the hypothesis that DNA molecules newly formed within cells are replicas of already existing molecules, thus ensuring the preservation and transmission from generation to generation of the biological information contained in their highly specific structure. The experimental evidence is, of course, of a preliminary nature only, and it requires confirmation and further development.

It will be evident from this short review that these discoveries made in the laboratories of the American biochemists S. Ochoa and A. Kornberg have opened up new prospectives for the study of the nucleic acids. The exceptional importance and significance of these discoveries have achieved wide recognition. One of the expressions of this recognition has been the award to S. Ochoa and A.Kornberg of a Nobel prize in 1959.

A. S. Spirin

RUSSIAN JOURNALS FREQUENTLY CITED
[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Émbriol.	Arkhiv Anatomii Gistologii i Émbriologii	Archives of Antomy, Histology, and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Archives of Biological Science USSR
Arkh. Patol.	Arkhiv Patologii	Archives of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanicheskii Zhurnal	Journal of Botany
Byull. Éksptl. Biol. i Med.	Byulleten Éksperimentalnoi Biologii i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologicheskii	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademii Nauk SSSR	Proceedings of the Academy of Sciences USSR
Éksptl. Khirurg.	Éksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rastenii	Fiziologiya Rastenii	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologicheskii Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenov Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademii Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografii	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichnii Zhurnal Ukrainskii	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichnii Zhurnal	Microbiology Journal
Nevropatol., Psikhyat. i Psikhogig.	Nevropatologiya, Psikhyatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologicheskii Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Éndokrinol. i Gormonoterap.	Problemy Endokrinologii i Gormonoterapii	Problems of Endocrinology and Hormone Therapy
Problemy Gematol.	Problemy Gematologii i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetskii Vrachebnyi Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology

*BRITISH-AMERICAN TRANSLITERATION SYSTEM.

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Abbreviation	Journal	Translation
Terap. Arkh.	Terapevticheskii Arkhiv	Therapeutic Archives
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademii Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestva Estestvoisp.	Trudy Leningrad Obshchestva Estestvoispytatelei	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Eksptl. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Meditsiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainskii Biokhimichnii Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademii Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgii imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biologii	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universitera, Seriya Biologii i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmologii	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenologii i Radiologii	Bulletin of Roentgenology and Radiology
Vestnik Venerol. i Dermatol.	Vestnik Venerologii i Dermatologii	Bulletin of Venereology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo SSSR	Wine-Making and Viticulture
Voprosy Klin.	Voprosy Klinicheskie	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimii	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusologii	Problems of Medical Virology
Voprosy Neirokhirurg.	Voprosy Neirokhirugii	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkologii	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhologii	Voprosy Psikhologii	Problems of Psychology
Voprosy Virusologii	Voprosy Virusologii	Problems of Virology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii	Journal of Microbiology, Epidemiology, and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatologii i Psikhiatrii imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
Zhur. Vysshei Nerv. Deyatel.	Zhurnal Vysshei Nervnoi Deyatelnosti imeni I. P. Pavlova	I. P. Pavlov Journal of Higher Nervous Activity
Zool. Zhur.	Zoologicheskii Zhurnal	Journal of Zoology

SIGNIFICANCE OF ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN SOVIET PERIODICALS

FIAN	Phys. Inst. Acad. Sci. USSR.
GDI	Water Power Inst.
GITI	State Sci.-Tech. Press
GITTL	State Tech. and Theor. Lit. Press
GONTI	State United Sci.-Tech. Press
Gosenergoizdat	State Power Press
Goskhimizdat	State Chem. Press
GOST	All-Union State Standard
GTTI	State Tech. and Theor. Lit. Press
IL	Foreign Lit. Press
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd. AN SSSR	Acad. Sci. USSR Press
Izd. MGU	Moscow State Univ. Press
LEIIZhT	Leningrad Power Inst. of Railroad Engineering
LET	Leningrad Elec. Engr. School
LETI	Leningrad Electrotechnical Inst.
LETIIZhT	Leningrad Electrical Engineering Research Inst. of Railroad Engr.
Mashgiz	State Sci.-Tech. Press for Machine Construction Lit.
MEP	Ministry of Electrical Industry
MES	Ministry of Electrical Power Plants
MESEP	Ministry of Electrical Power Plants and the Electrical Industry
MGU	Moscow State Univ.
MKhTI	Moscow Inst. Chem. Tech.
MOPI	Moscow Regional Pedagogical Inst.
MSP	Ministry of Industrial Construction
NII ZVUKSZAPIOI	Scientific Research Inst. of Sound Recording
NIKFI	Sci. Inst. of Modern Motion Picture Photography
ONTI	United Sci.-Tech. Press
OTI	Division of Technical Information
OTN	Div. Tech. Sci.
Stroiizdat	Construction Press
TOE	Association of Power Engineers
TsKTI	Central Research Inst. for Boilers and Turbines
TsNIEL	Central Scientific Research Elec. Engr. Lab.
TsNIEL-MES	Central Scientific Research Elec. Engr. Lab.—Ministry of Electric Power Plants
TsVTI	Central Office of Economic Information
UF	Ural Branch
VIESKh	All-Union Inst. of Rural Elec. Power Stations
VNIIM	All-Union Scientific Research Inst. of Meteorology
VNIIZhDT	All-Union Scientific Research Inst. of Railroad Engineering
VTI	All-Union Thermotech. Inst.
VZEI	All-Union Power Correspondence Inst.

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. — Publisher.

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